Cortisol and conservation: Understanding barriers to the recovery of a critically endangered species using stress physiology

by

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GENERAL ABSTRACT

Chronic exposure to high levels of glucocorticoid hormones can be detrimental to survival and reproduction. Captive breeding and release may inadvertently subject animals to conditions that elicit an increase in glucocorticoids, if conditions in captivity or habitats in the wild are inadequate. The critically endangered Vancouver Island marmot (*Marmota vancouverensis*) was used to investigate variability in hair cortisol concentration, a measure of systemic glucocorticoid exposure. Marmots were sampled from the captive, captive-release and wild populations. Captive animals had significantly lower cortisol levels than wild animals. Several marmots were also sampled from the historic wild population that lived on Vancouver Island prior to the proliferation of anthropogenic disturbances. A comparison between contemporary and historic animals showed that marmots at most colonies had cortisol levels that were the same as historic level. Three colonies had significantly elevated cortisol levels. Cortisol concentration in the wild was best explained by proximity of a colony to logging roads.

Key words: endangered mammals; hair cortisol analysis; land use; forestry; conservation; linear mixed modeling

DEDICATION

This thesis is dedicated to my beautiful grandmother, Jean M. Acker, from whom I undoubtedly inherited my love of animals.

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GENERAL INTRODUCTION

The mammalian hypothalamic-pituitary-adrenal axis (HPA axis) is an endocrine system involved in allostasis, an ongoing process which maintains an organism's internal steady state or homeostasis in the face of disturbance (McEwen and Wingfield, 2003). Activation of the HPA axis is a key part of the stress response, a transient physiological state which mobilizes an animal's life-saving resources when faced with the stressor of a perceived threat or a challenging environment (Reeder and Kramer, 2005). An individual that is unable to avoid a threat or find a suitable environment may fail to re-establish homeostasis (Boonstra, 2013). This can result in a state of allostatic overload wherein an individual's overtaxed physiological systems have deleterious effects on their own fitness (McEwen and Wingfield, 2003). Extensive research on laboratory rodents, and biomedical studies centred on human health, have suggested that allostatic overload leads to a breakdown of processes that are vital for immune function (Dhabhar, 2000; Padgett and Glaser, 2003), reproductive success (Hardy *et al.*, 2005; Wiebold *et al.*, 1986) and memory formation (Liston *et al.*, 2013). In light of these findings, conservation biologists have sought to understand allostasis in endangered species, both in captive populations and in intensively-managed, free-ranging populations that are vulnerable to extinction (Kersey and Dehnhard, 2014).

One can assess HPA activity directly by measuring hormone levels and indirectly by measuring such indices as body condition and behavior (Sheriff *et al.*, 2011). Hormones involved in allostasis include corticotropin releasing hormone (CRH), secreted by the hypothalamus; adrenocorticotropic hormone (ACTH), by the pituitary gland; and glucocorticoids (GCs), by the cortex of the adrenal gland (Reeder and Kramer, 2005). This latter group includes corticosterone, cortisone and cortisol and of these, corticosterone dominates in small rodents, while cortisol is the primary GC in most other mammals (Sheriff *et al.*, 2011). GCs can be detected in saliva and serum, and their metabolites are found in the urine and feces of most mammal and bird species (Touma and

Palme, 2005). However, using these matrices presents several challenges when analyzing stress in free-ranging animals, particularly when chronic GC exposure is of primary interest. Collecting these substrates requires researchers to have close-range access to animals, often trapping and handling them. Not only can this confound results (e.g., Harms *et al.*, 2012), but it is time-consuming and costly. Furthermore, these measurements are point estimates of GC levels over the course of minutes or hours. Thus, determining long-term shifts in GC levels, which characterise chronic rather than acute conditions, can be challenging (Dickens and Romero, 2013).

Glucocorticoids are also present in hair and feathers, and the use of these matrices to quantify GCs may allow field researchers to overcome the drawbacks of more traditional methodology (Koren *et al.*, 2002). Hair and feather samples can be collected with little to no direct contact between researcher and animal (Koren *et al.*, 2002). They also offer insight into long-term patterns in HPA activity, accounting for the integration of GCs into the keratin structure as it grows over the course of weeks or months (Kirschbaum *et al.*, 2009). They are relatively stable. Macbeth et al. (2010) found that storage temperature of hair samples had no effect on hair cortisol levels, nor did exposure to the elements (including rain, snow and sunlight) over the course of 18 days prior to collection. Thus samples can be collected, transported and stored with few of the special considerations given to other matrices that are logistically challenging during field studies (e.g. urine (Miki and Sudo, 1998); feces, (Terio *et al.*, 2002)). Additionally, evidence from human burial sites suggests that hair will remain stable over considerable periods of time (>500 years, (Webb *et al.*, 2010)). These findings suggest that researchers may be able to use hair samples collected opportunistically from such diverse sources as historic pelage, non-invasive hair snares and commercial fur repositories. This may prove to be particularly useful in studying small, endangered populations as well as elusive species (Sloane *et al.*, 2000).

GC levels fluctuate in response to a wide variety of stimuli and are also related to many factors intrinsic to an individual (Dantzer *et al.*, 2014). Elevated GC levels in free-ranging organisms are likely an adaptive response to many circumstances that arise in natural environments, such as when food availability fluctuates considerably and individuals must be able to survive on limited energy reserves (Adamo, 2012). This may explain why no consistent relationship exists between high GC levels and poor fitness in natural populations (Bonier *et al.*, 2009). However, the fitness of captive animals, as well as the challenges they face in captive environments, may be more akin to laboratory animals than free-ranging conspecifics, as captive populations that live in artificial environments are simultaneously released from the pressures of natural and sexual selection (Lynch and O'Hely, 2001) and subject to a variety of challenges (interactions with humans, transportation between captive facilities, constraints on natural behaviour) that are relatively novel on an evolutionary timescale (Morgan and Tromborg, 2007). Indeed some species respond to the challenges of captivity by upregulating GC signalling (Jachowski *et al.*, 2012; Scott *et al.*, 2017; Wells *et al.*, 2004) and high GC exposure is likely related to displays of stereotypy, difficulty reproducing and poor health that are reported in some captive populations (Clubb and Mason, 2003; Mason, 2010; Ralph *et al.*, 2016; Wielebnowski *et al.*, 2002). The survival and reproduction of captive populations that are kept in captivity for conservation purposes (i.e. endangered species) is critical. HPA activity may be one of several valuable metrics to measure responses to captivity and thus determine individuals or populations that fare poorly in a captive environment (Palme, 2012)

The maintenance of captive populations may be a part of a species recovery plan involving captive breeding and release of individuals to the wild. As such, conservation success will be dependent on both sufficient reproduction in captivity and adequate habitat to support a free-ranging population (Kleiman, 1989). Habitat loss and fragmentation are commonly cited as threats to biodiversity (Fahrig, 2003; McCune *et al.*, 2013; Schipper *et al.*, 2008). These terms describe use of

the biosphere by humans at the expense of other species such as when human activity is initiated on or near habitat patches that must be subsequently abandoned by native species (Sawyer *et al.*, 2006) or which become degraded and cease to support robust populations (Bender *et al.*, 1998; Gibson *et al.*, 2013). Anthropogenic disturbances are associated with small to moderate increases in baseline GC levels in wildlife (Dantzer *et al.*, 2014), perhaps because individuals living in disturbed habitats must upregulate GC signalling to overcome the challenges associated with poor habitat (Bonier *et al.*, 2009). This may provide wildlife managers with a signal as to which habitat patches support animals that perceive many challenges in their environment relative to other potential release sites where animals may face fewer challenges and have lower baseline GC levels (Homan *et al.*, 2003). This has an immediately practical application, as managers must make decisions as to where animals are released and translocated and may wish to take into account the degree to which one habitat patch challenges individuals more than another. Furthermore, analysis of patches where GC levels are relatively high may identify features of these habitats that are common and may be related to the challenges that animals there perceive.

This study employs hair cortisol analysis to quantify both modern and historic levels in a species-at-risk, in an attempt to highlight potential problem areas in the species' management including the role of captivity and habitats that may no longer serve the species' needs. The Vancouver Island marmot is a medium-sized species of ground squirrel that lives in small family groups or colonies (Heard, 1977). Marmot colonies are found in subalpine meadows in the southern and mid-island mountain ranges of Vancouver Island, a large island on the Pacific coast of Canada (Bryant and Janz, 1996). Approximately 300 marmots currently live in the wild, distributed in colonies across a 1000 km^2 area (Jackson *et al.*, 2015). Due to its small and fragmented population, the Vancouver Island marmot is listed as critically endangered by the International Union for the

Conservation of Nature (IUCN) (Roach, 2017). Remarkably, many threats to survival that other species face appear to be absent.

Vancouver Island marmots are one of three marmot species of interest on the IUCN Redlist, along with the Menzbier's marmot (*Marmota menzbieri,* IUCN category: Vulnerable) and the tarbagan (*Marmota siberica,* IUCN category: Endangered). The threats to the latter two species have been clearly defined. The Menzbier's marmots are threatened by conversion of their habitat to agricultural land (Tsytsulina, 2008). The tarbagan is exploited as a game species and is also affected by outbreaks of plague caused by *Yersinia* sp. (Clayton, 2016). By contrast, most Vancouver Island marmot colonies are far from arable land and are thus marginally impacted by growth in the demand for agriculture. The species is also legally protected from exploitation for human use, and cases of *Yersinia* infection are very rare (McAdie, 2004). The Vancouver Island marmot's high altitude habitat also keeps it away from direct conflicts with invasive species, another major threat to many endangered species (McCune *et al.*, 2013). For example, the Eastern cottontail rabbit (*Sylvilagus floridanus*), an introduced species on Vancouver Island that might outcompete marmots for food, is not found above 400m, well below the range of the marmots (Bertolino *et al.*, 2011). Like the Alaskan marmot (*Marmota broweri*) that inhabits remote, montane habitat in North America (Cassola, 2016), the Vancouver Island marmot should be a relatively stable species.

In addition to facing few identifiable threats, considerable efforts have been made to prevent the species extinction. A captive breeding and release program has been operating in Canada since 1997 and is currently supported by two zoos and administered by the Marmot Recovery Team (Jackson *et al.*, 2015; McAdie, 2004). Nonetheless, the species' recovery remains precarious. The aim of this study is to analyze some potential challenges that Vancouver Island marmots face to try and identify risks to the species survival. Vancouver Island marmots are likely to share some

characteristics of matrilineal cooperative breeders, the breeding strategy used by many other marmot species (Armitage, 2014). In some species of cooperative breeders, such as meerkats (*Suricata suricatta*) and Alpine marmots (*Marmota marmota*), females may be able to control the breeding success of conspecifics through agonistic behaviours that induce a stress response (Hackländer *et al.*, 2003; Young *et al.*, 2006). The stress response suppresses reproduction by acting on the hypothalamic-pituitary-gonadal axis, indicating a direct link between fitness and stress (Wasser and Barash, 1983). If Vancouver Island marmots are also subject to reproductive suppression in the face of chronically elevated GC levels, as Armitage (2014) suggests, then determining if and when GC levels are high in this species is vital to improving outcomes of the captive breeding and release program and ensuring the species survival into the future.

This thesis is divided into three chapters. The first is a description of various tests that were necessary in order to better understand the physiological validity of hair cortisol measurements. This includes a test of the validity of hair cortisol measurements that are taken from specimens housed in natural history collections which may have been subjected to treatment with harsh preservatives. The second chapter is a description of cortisol levels among marmots living in different conditions. Broadly, it examines differences between marmots in captivity and in the wild, including those that are born in captivity and released to the wild. The third chapter examines differences within captive and wild marmots that occupied different colonies in space and time. This chapter also describes an attempt to explain the differences between colonies which might account for the variable cortisol levels.

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CHAPTER ONE – The effects of body region, season and external arsenic application on the concentration of cortisol in hair

Abstract

Hair cortisol analysis has been used to quantify hormone levels in circulation within several mammal species. Hair remains stable for decades or centuries, allowing researchers to use archived hair samples to investigate hormone levels that span long time periods. However, several studies have found that intra-individual variability, driven by the body region from which a sample is derived, confounds measurements of systemic glucocorticoid hormone concentrations. In addition, the external application of chemical agents to hair can remove or concentrate molecules of interest. These may preclude the use of samples that have been collected opportunistically and/or those which have been housed in museum collections. Using a captive population of Vancouver Island marmots (*Marmota vancouverensis*), I found a strong effect of body region on the concentration of cortisol within hair, as well as an effect of season, suggesting that intra-individual variability is not stable through time. The reason for these seasonal effects is not clear and further study is necessary. Researchers using samples from an unknown body region should exercise caution in interpreting their results. Using a collection of American mink (*Neovison vison*) pelts, I found that application of the preservative arsenic in the form of a soap does not cause a significant decrease in cortisol. This suggests that samples held in museum collections can be used to quantify cortisol.

Introduction

Hair can be used as a substrate with which to quantify molecules found in the circulatory system of mammals, including hormones (Koren *et al.*, 2002; Yang *et al.*, 1998), metals (Bocharova *et al.*, 2013; Soba□ ska, 2005) and xenobiotics (Baumgartner *et al.*, 1989; D'Havé *et al.*, 2005). The glucocorticoid stress hormones cortisol and corticosterone can be found in hair samples and have been quantified in captive (Carlitz *et al.*, 2015; Dettmer *et al.*, 2012; Malcolm *et al.*, 2013), free-ranging (Cattet *et al.*, 2014; Koren *et al.*, 2008) and domesticated (Bennett and Hayssen, 2010; Finkler and Terkel, 2010; González-de-la-Vara *et al.*, 2011) mammals; these studies have shown correlations between hair cortisol and traits such as reproductive and social status, health and body condition and abnormal behaviours (e.g. self-injurious behaviour). Recently, several validation studies have shown that a single ACTH injection does not lead to increased hair cortisol in captive ungulates (*Rangifer tarandus granti* and *R. tarandus tarandus*, (Ashley *et al.*, 2011)) but that repeated ACTH challenge does lead to increased hair cortisol levels in captive Canada lynx (*Lynx canadensis*, (Terwissen *et al.*, 2013)) and free-ranging chipmunks (*Tamias striatus*, (Mastromonaco *et al.*, 2014)). Similarly, Yu et al. (2015) found increased levels of corticosterone in the hair of mice subjected to a social defeat paradigm, an experimental design intended to induce chronic stress. Taken together, these findings suggest that glucocorticoid (GC) levels in hair accurately reflect long-term changes in HPA activity and that these changes are related to systemic glucocorticoid levels. The use of hair analysis to quantify systemic exposure is advantageous for two reasons. First, because hair grows over the course of weeks or months, hair analysis provides an opportunity to measure physiological changes on this same time scale (Kirschbaum *et al.*, 2009; Noël *et al.*, 2015). With hair analysis, it is possible to detect chronic exposure to toxins (Boumba *et al.*, 2006; D'Havé *et al.*, 2005) and to detect hormonal shifts associated with disease or pregnancy (Kirschbaum *et al.*, 2009; Thomson *et al.*, 2010; Wester and van Rossum, 2015). It can otherwise be difficult to measure these phenomena using substrates such as

blood or saliva because measurements made using these substrates are sensitive to the circadian rhythms of metabolism (Price *et al.*, 1983; van Cauter *et al.*, 1996) and repeated point estimates are necessary to establish a pattern of exposure. Second, using hair samples it is possible to make a retrospective estimate of an individual's exposure to extrinsic contaminants or GC hormones, as hair analysis has yielded biologically valid concentrations of metals (Bocharova *et al.*, 2013; Lewin *et al.*, 1982) and GCs (Macbeth *et al.*, 2010; Webb *et al.*, 2010) in hair samples that are several decades or centuries old.

Despite these benefits, hair as a substrate has several drawbacks that must be addressed. Firstly, hair cortisol concentration is known to vary within an individual depending on the body region from which the sample is drawn (Ashley *et al.*, 2011; Carlitz *et al.*, 2015; Macbeth *et al.*, 2010). This effect appears to be species-specific, therefore a test for the relationship between body region and hair cortisol concentration should be undertaken before interpreting cortisol values drawn from hairs that have been collected opportunistically. It is not clear why hair cortisol varies by body region. Carlitz et al. (2015) investigated the possibility that it may be due to blood flow at the skin which varies at different parts of the body and therefore delivers variable amounts of cortisol to the hair follicle. They found some evidence for this in a study of chimpanzees, *Pan troglodytes*, but were not able to explain hair cortisol concentration in the shoulder using temperature alone, as they could with other body regions. They suggest that cortisol may be "washed out" of hair by water, ultraviolet radiation or other external factors. When human hair was subjected to a hot water bath (40-80 degrees Celsius), a shampoo treatment or 9 hours of ultraviolet irradiation *in vitro,* cortisol concentration in the hair decreased (Li *et al.*, 2012). However, hair samples derived from many free-living wild animals are not likely to be subjected to such conditions. Moreover, Macbeth et al. (2010) did not find a weathering or washout effect in grizzly bear (*Ursos arctos*) hair that was left in experimental hair snares exposed to the elements for up to 18 days. To my knowledge, despite

several studies on changing hair cortisol concentration along the hair shaft, no published studies have measured hair cortisol concentration at different body regions through time to determine whether relative cortisol concentrations at each location are static within an individual. I measured hair cortisol concentration at five body region during two seasons to establish the pattern of intra-individual variation in Vancouver Island marmots (*Marmota vancouverensis*). I expected body regions to be significantly different, but that this would not vary by season. If so, this would support to Carlitz et al.'s (2015) hypothesis that explains hair cortisol concentration as a function of an individual's physiology.

Another challenge to interpreting hair cortisol concentrations is that hair can be altered by external application of chemicals. It is well-known in the forensic science community that results of human hair analysis can be distorted by cosmetics. A shampoo and a commercial dye, for instance, were both shown to remove opiate drug metabolites from human scalp hair (Jurado *et al.*, 1997). By contrast hair moisturizing or conditioning products may cause hair to absorb and retain xenobiotics from the environment (Kidwell *et al.*, 2015). In both cases, external application of chemical adulterants leads to unreliable results regarding systemic exposure. Although most non-human animals do not have their hair treated with cosmetic chemicals, specimens that are kept in natural history collections may be subjected to chemical treatment and it is unclear how these might affect the concentrations of hormones or toxins within the hair (Bocharova *et al.*, 2013). Fumigants and poisons were used throughout the 1800s and early 1900s by museums to preserve their natural history collections, including study skins. Among these are arsenic, mercury, tobacco and camphor which may have been used as recently as the 1960s (Goldberg, 1996). It may not always be clear which specimens have received which treatment and not all museums have the resources to test their collections in full (e.g.., The Victoria and Albert Museum in London (Cullen, 2008)). It is reasonable to assume that all taxidermy specimens collected before 1960 may have been treated at

some point with a toxic substance such as arsenic (Marte *et al.*, 2006). Fortunately, many museum collectors and preparators left behind detailed instructions on the preparation of study skins for museums. As such, it is possible to recreate the treatments outlined in these documents to conduct an experimental test for the effects of preservatives on the detection of hormones or other molecules within hair samples. In 1965, R. M. Andersen at the National Museum of Canada (now known as the Canadian Museum of Nature), published the fourth edition of Methods of Collecting and Preserving Vertebrate Animals. In it, he mentions such chemicals as alum, borax, naphthalene, benzene and "arsenical soap" as treatments for mammal pelts. Of these, only borax is still in use today in major Canadian museums (J. Miller, Royal Ontario Museum, pers. comm.) In order to understand how some of these chemicals may alter hormone levels in fur, I conducted an experiment designed to recreate the arsenical soap treatment espoused by Andersen. I measured cortisol levels in fur before and after pelts were treated with arsenical soap, prepared and applied to the skin as per Andersen (1965), with consideration of the writings of Schmidt (1824). I expected treatment with soap to have a significant wash-out effect on hair cortisol concentration.

Methods

Experiment 1: Body region and Season

Study animals

A population of Vancouver Island marmots (*Marmota vancouverensis*), housed at the Toronto Zoo, were used to determine if there is an effect of body region or season on hair cortisol concentration. In Vancouver Island marmots, moulting occurs in July, beginning with adult males and non-reproductive females, followed by young adults, yearlings and finally females that have weaned a litter and their young (Naughton, 2014). I collected hair samples from five different body regions (hind limb, chest, forelimb, rump and back) in late March and late August, to compare hair cortisol

concentrations after a period of hibernation and concentrations immediately following the moult in active marmots. Vancouver Island marmots were housed in a breeding facility that is not accessible to the public. With the exception of one yearling marmot, all marmots were housed in breeding pairs. Marmots hibernate from October until April and emerge from their burrows in May. Hibernating marmots were sampled in late March without the use of anaesthetic. A second sample was taken in late August when marmots were anaesthetized for routine veterinary care using isofluorane gas. This procedure was approved by the Toronto Zoo's Animal Care and Research Committee (REF No. 2016-03-01).

Sample collection

A 2 cm x 2 cm patch of fur was shaved from the left hind limb, back, rump, chest and right forelimb of each animal using clippers to cut as close to the skin as possible. Clippers were cleaned using 70% isopropanol and compressed air between samples. Samples were collected in plain white envelopes and stored at room temperature until use.

Hair cortisol analysis

The hair cortisol extraction protocol was adapted from Mastromonaco et al. (2014) and Majchrzak et al. (2015). Briefly, the hair was cut into segments less than 0.5 cm in length and weighed in glass scintillation vials. To wash each sample, 0.75 ml of 100% methanol was added and the sample was vortexed for 10 seconds. The wash liquid was pipetted off and the vial was left open for 5 minutes for remaining methanol to evaporate. Cortisol was extracted from washed samples during a 24 hour incubation in 100% methanol on an orbital shaker (MBI Orbital Shaker; Montreal Biotechnologies, Montreal, Canada). Following incubation, samples were spun in a centrifuge at 3500 rpm for 10 minutes. Hair was discarded and 1500 μL of extract was pipetted into a new vial and dried in a fume hood under constant air flow for 24-48 hours.

Dried extracts were reconstituted by adding 150 μL of phosphate buffer (0.1mM sodium phosphate, pH 7.0, 9 g of NaCl and 1 g of bovine serum albumin per liter) and vortexing for 10 seconds. Cortisol values were determined using an EIA that has been previously described by Terwissen et al. (2013) with modifications made by Majchrzak et al. (2015). Cortisol antiserum (R4972, C. Munro, University of California, Davis, USA) was diluted 1:12 000 in coating buffer (50 mM bicarbonate buffer, pH 9.6) and horseradish peroxidase anti-immunoglobulin (C. Munro, University of California, Davis, USA) was diluted 1:34 000 in phosphate buffer. Molecules that are cross-reactive to the cortisol antibody are: cortisol (100%), prednisolone (9.9%), prednisone (6.3%), cortisone (5%), corticosterone (0.7%), 21-deoxycortisone (0.5%), deoxycorticosterone (0.3%). Inter-assay CVs were calculated by running external controls at 25% and 65% binding in duplicate on each plate. The CV for high control (25% binding) was 9.2% and for low control (65% binding) was 5.6%. Along with monitoring the CV of each duplicate, intra-assay CVs were further evaluated by loading a pooled fecal extract diluted to 50% binding repeatedly across the plate. For this assay, the intra-assay CV was 3.6%. Cortisol standards used were 0.078 – 20 ng/ml = 78 – 20,000 pg/ml (Steraloids Inc., Newport, USA; cat # Sigma H-0135).

Microtitre plates were incubated overnight at 4 degrees Celsius with 50 μL per well of cortisol antiserum in coating buffer. Plates were washed with a 0.02% Tween 20 solution using a microplate washer (BioTek Instruments, Winooski, USA) and 50 μL of reconstituted hair extract, standard or control were pipetted into the plate in duplicate. This was followed immediately by 50 μL per well of horseradish peroxidase in phosphate buffer. Plates were further incubated for 2 hours at room temperature and washed again using a 0.02% Tween 20 solution. Finally, 100 μL of substrate solution was added to each well (50 mM citrate, 1.6 mM hydrogen peroxide, 0.4 mM 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid), ph 4.0) and plates were incubated a final time for 30 to 45

minutes at room temperature. Absorbance at 405 nm was measured using a spectrophometer (Dynex Technologies, Chantilly, USA).

Statistical analyses

All data were transformed with the natural logarithm to meet assumptions of normality (see Appendix A for further information on transformation). A mixed ANOVA was used to determine the effect of body region and season on hair cortisol concentration. Tukey's HSD was used to determine main effects of body region or season on hair cortisol concentration, and any changes in body region with time. The statistical test and transformation were performed using R statistical software (R Core Team, 2015).

Experiment 2: Museum treatment

Pelt preparation

To test the effects of arsenical soap, twelve American mink (*Neovison vison*) were collected from a fur farm in Southern Ontario, Canada. They were presumed dead from natural causes and were found by farm personnel in their cages on the mornings of July 5 and 6, 2016. All remains were stored at -20 degrees Celsius and were gradually brought to 4 degrees Celsius before being skinned and scraped to remove excess tissue. Pelts were hung to dry fur side out in a fume hood on a wooden board, a wire rack or a plastic rack. Drying times varied from 24-48 hours.

Arsenical soap preparation

Arsenical soap was prepared following the recipe used by the field naturalists with the Canadian Museum of Nature (Andersen, 1965). In a fume hood, 113.5 g of a white laundry soap bar (sodium tallowate, sodium cocoate, sodium palm kernelate, glycerin; The Soap Works, Toronto, Canada) was melted in 75 ml of distilled water over low heat, stirred regularly. Once soap had melted to a thick liquid, 21.25 g of potassium bicarbonate ($KHCO_3$, Cas Brewhouse, Sudbury, Canada), 113 g of white arsenic (As_2O_3, Fisher Scientific, Hampton, USA), 20 ml of pure essential camphor oil

(*Cinnamomum camphora*; Puresource Inc., Guelph, Canada) and 10 mL of 95% ethanol were added. The solution was stirred until it reached uniform consistency, poured into a clean, glass Mason jar (Bernardin, Toronto, Canada) and left overnight to re-solidify.

Sampling and treatment of skins

Arsenic-free samples were taken from the left rump of each skin and arsenic-treated samples were taken from the right rump (as in Ashley et al. 2011). A small, 2 cm x 2 cm patch of fur was shaved using a beard trimmer (Maxtrim Model GMT17SDMC; Conair, East Windsor, USA) to cut as close to the skin as possible. Each pelt was washed using arsenical soap (Andersen, 1965). Specifically, 0.01g of arsenical soap and 1.5mL of distilled water were lathered using a paint brush. The lather was applied to the skin, first on the leather, then on the fur (Schmidt, 1824). The lather was worked into the skin using the brush for 10 seconds on each side and the skin was then rinsed liberally with distilled water. All skins were left to dry overnight in the fume hood. Once dry, each pelt was sampled a second time to obtain an arsenic-treated sample.

Hair cortisol analysis

The samples were analyzed as described above.

Statistical analysis

All hair cortisol concentrations were transformed with the natural logarithm to meet assumptions of normality (see Appendix A for further information on transformation). A paired two-tailed t-test was used to compare hair cortisol concentration in mink pelts before and after arsenic treatment. The statistical test and transformation were performed using R statistical software (R Core Team, 2015).

Results

Experiment 1: Body region and Season

A total of 8 marmots living in the Toronto Zoo were sampled in both March and August. One marmot that was sampled in March died before sampling in August. This marmot was 10 years old and in poor health so these data were omitted from analysis. The sample included data from 3 females and 5 males ranging in age from less than one year to 7 years. A mixed ANOVA showed that there was a significant main effect of body region on hair cortisol concentration ($F = 6.373$; $df = 4, 63$; $p = 0.000226$), a significant main effect of season ($F = 17.572$; $df = 1, 63$; $p < 0.0001$) and a significant interaction ($F = 4.573$; $df = 4, 63$; $p = 0.00263$). The left hind limb was significantly different from the right fore limb (Tukey HSD: 1.03231; $p = 0.0411$), the rump (Tukey HSD: 1.04495; $p = 0.0373$) and the back (Tukey HSD: 1.23038; $p = 0.0076$) if season was held constant. Cortisol measurements in the chest decreased significantly between March and August (Tukey HSD: 1.81595; $p < 0.01$) (see figure 1.1).

Experiment 2: Museum treatment

Twelve mink pelts were sampled before and after arsenic treatment for a total of 24 paired observations. Of these, 23 measurements were above reliable detection limits. The missing observation came from mink number 12. This mink was removed from the data set, yielding a total of 22 observations from 11 mink pelts. A paired t-test revealed that there was no significant difference between hair cortisol concentration following arsenic treatment ($t = -1.5206$, $df = 10$, $p = 0.1593$) (see fig 1.2).

Discussion

The hair cortisol concentration in Vancouver Island marmot (*M. vancouverensis*) hair varied with body region. Regardless of season, the hair cortisol concentration in the left hind limb was significantly

higher than in the right forelimb, the rump or the back. Samples from the back were taken from the upper back between the shoulder blades. Thus the finding that the rump and back had similar hair cortisol concentration in Vancouver Island marmots is similar to the results reported by Ashley et al. (2011) who found that the hair cortisol concentration in the rumps and shoulders were similar within caribou (*R. tarandus granti*) and reindeer (*R. tarandus tarandus*) and Macbeth et al. (2010) who found similar cortisol concentrations in rump and shoulder within grizzly bears (*U. arctos*). However, Macbeth et al. (2010) also found that hair on the abdomen of grizzly bears had a similar cortisol concentration to the back and rump; my results showed that hair from the ventral side of Vancouver Island marmots had a different cortisol concentration from the rump and shoulder in both the spring and the summer, but that the direction of the relationship changed. That is, in March the hair from the chest had a high cortisol concentration, more similar to that of the hind limb, while in August the cortisol concentration was significantly lower and was more similar to that of the forelimb. This pattern observed in the marmots from March is similar to the one observed by Carlitz et al. (2015) who found that hair cortisol concentration in chimpanzees (*P. troglodytes*) was higher in the chest than the back or the forelimb. Together, these findings highlight the importance of controlling for body region whenever possible (Terwissen *et al.*, 2013) and demonstrate further evidence that there is marked species-specific variation of hair cortisol concentration within individuals.

To my knowledge, the results that show two patterns of hair cortisol concentration within the same individuals sampled at different times of the year represent a novel finding. In March, hair cortisol concentration was high in the hind limb and chest and it was lower in the forelimb, rump and back. In August, hair cortisol concentration remained relatively high in the hind limb, but became more similar to the rump and back while the chest and the right forelimb had a lower hair cortisol concentration. A mixed ANOVA showed that the two seasons were significantly different

from each other. Because the samples in March 2016 and August 2016 represent hair growth from the July 2015 and July 2016 moulting periods respectively, one could attribute that difference to different systemic cortisol levels among the marmots from one year to the next based on changes to their environment. However, the significant interaction term between body region and season suggests that some of the difference between seasons is driven by the partitioning of cortisol to different body regions from one sampling time to the next. These results do not lend strong support to the hypothesis that internal physiological mechanisms are solely responsible for the observed differences in hair cortisol concentration at different body regions. These results are evidence that cortisol is not deposited consistently in the hair of a given body region within an individual. If hair cortisol concentration is dependent on blood flow to a given area during hair growth, as Carlitz et al. (2015) proposed, there would have to be some mechanism to differentially direct blood flow away from or to the chest during a marmot's moult. It is feasible that marmots may lose excess body heat by redirecting blood flow to the ventral skin surface during periods of intense heat. In Alpine marmots (*Marmota marmota*), laying prone on rocks or soil during the heat of the day is a thermoregulatory behaviour that helps individuals lower their body temperature (Turk and Arnold, 1988). The coat is thinner on a marmot's ventral side and thus it is likely easier to lose heat to the environment this way. However, the Vancouver Island marmots at the Toronto Zoo are given access to climate controlled conditions which negates the need for such behaviour. Furthermore, average temperatures in July 2016 (23.8 degrees Celsius, Toronto City weather station, Environment Canada) were higher than July 2015 (21.9 degrees Celsius, Environment Canada) so that if marmots were spending their time outside of the climate controlled enclosures, they would have had to lose more heat in 2016 than they did in 2015 which is not consistent with higher hair cortisol in ventral samples in 2015.

Identifying a mechanism for these results is outside the scope of this study. Nonetheless, these results indicate that in addition to controlling for body region, it is important to select a body region wherein hair cortisol concentration accurately reflects systemic cortisol. In Vancouver Island marmots, it is clear that if one consistently sampled from the chest, one would arrive at a different conclusion regarding systemic cortisol levels than if one consistently sampled from the hind limb. The hair cortisol concentration in the chest declined significantly between sampling periods, while the hindlimb, forelimb and back all declined slightly and the rump increased slightly. Given the concordance between the difference in hair cortisol measured in the hindlimb, forelimb and back, these may be appropriate candidates for sampling areas in future marmot studies and a study designed to investigate the correlation between sampling sites within a marmot should include these regions. These results would benefit considerably from a biological validation. Ashley et al. (2011) took body region variability into consideration in the design of their ACTH challenge in reindeer and caribou, however they failed to induce a state of chronic stress in their animals by injecting them a single time with the ACTH analogue. Those who undertake future validation studies with a longer time course for injections (as in (González-de-la-Vara *et al.*, 2011; Terwissen *et al.*, 2013)) should investigate this phenomenon more thoroughly by sampling from several body regions before and after ACTH challenge.

The application of arsenical soap to mammal skins as described by R M Andersen (1965) did not cause hair cortisol concentration to change in the short-term. This suggests that museum samples should not be discounted as valid sources data regarding historical cortisol levels, even if they are potentially contaminated with arsenic. It remains a possibility that I have only detected a short-term effect and that long-term contact with small amounts of arsenical soap residue have an effect on hair cortisol concentrations. It would be possible to test the long-term effects of arsenic on cortisol concentration by sampling both specimens that test positive for arsenic and those that test

negative for arsenic and have been in collection for many decades, but I was not able to investigate this during the course of the research described here. It is also possible that I failed to detect a change in cortisol levels due to the small sample sizes used in this test. Figure 1.2 demonstrates a possible trend toward a decrease in hair cortisol concentration after treatment which may be indicative of a true decrease in cortisol between samples. Given the variance in the cortisol concentration measured in the untreated mink hair (SD = 0.701), a power analysis suggests that I would have only been able to reliably detect a large effect size (d = 0.8) using this data set (n = 11, power = 0.67), despite using a paired design. Thus it is possible that a small to moderate effect size was present.

It is also possible that the decline in cortisol between treatments was an artifact of surface contamination as the samples taken before the treatments may have been more contaminated than I anticipated so that a single methanol wash was not sufficient to clean them. The one-wash protocol was adapted from a protocol used to wash samples that were clipped from living animals. Remains of farmed mink, which were housed at high density and may have died due to some illness, are likely to have considerably more surface contamination than a healthy, living animal. Macbeth et al. (2010) found that it took two 3-minute washes in methanol to remove contamination from heavily soiled grizzly bear samples. All the samples taken after treatment would have been subject to a second wash as part of the treatment procedure and so would have been free of excess contamination. This might account for the discrepancy between the cortisol measured in the before and after treatment samples, although it is not statistically significant. Examination of the raw data shows that 9 of the 11 pairs show small to moderate changes in cortisol. Among four of the pairs, cortisol increased slightly, with a mean difference of 0.317 following treatment. Among five of the pairs, cortisol decreased slightly with a mean difference of -0.362 following treatment. The difference observed in figure 1.2 may therefore be driven largely by the results from two mink: #7 and #11. The

differences in cortisol before and after treatment of these pelts were -1.15 and -1.16, respectively. It seems plausible that these samples which proved particularly responsive to the treatment had excessive surface contamination that I did not adequately address with my protocol.

The arsenical soap treatment represents a strong preservative and was selected for the test on this basis. It is composed not only of arsenic, but of camphor (another preservative) and soap which has been implicated in washing compounds out of human hair (Jurado *et al.*, 1997). Furthermore, this preservative is applied using the mechanical force of a paintbrush to produce a lather. In contrast, preservatives such as tobacco, by virtue of being a fumigant, are applied passively. The estimates of hair cortisol concentration following treatment with the arsenical soap as I have described are likely very conservative compared to estimates one might make following a preservative treatment that is less taxing on the specimen. This technique is also historically accurate. Not only was the soap prepared in the same manner as Andersen (1965) described, but the skins were also removed and dried as he suggested. This ensured that the pelts were treated in a manner that approximated the field techniques of naturalists and curators in the early 1900s, as opposed to fur trappers and hunters whose purposes were certainly different. As such, in the third chapter of this thesis I will describe the use of Vancouver Island marmot pelts from several natural history collections to estimate mean cortisol levels in marmot colonies from approximately one hundred years ago.

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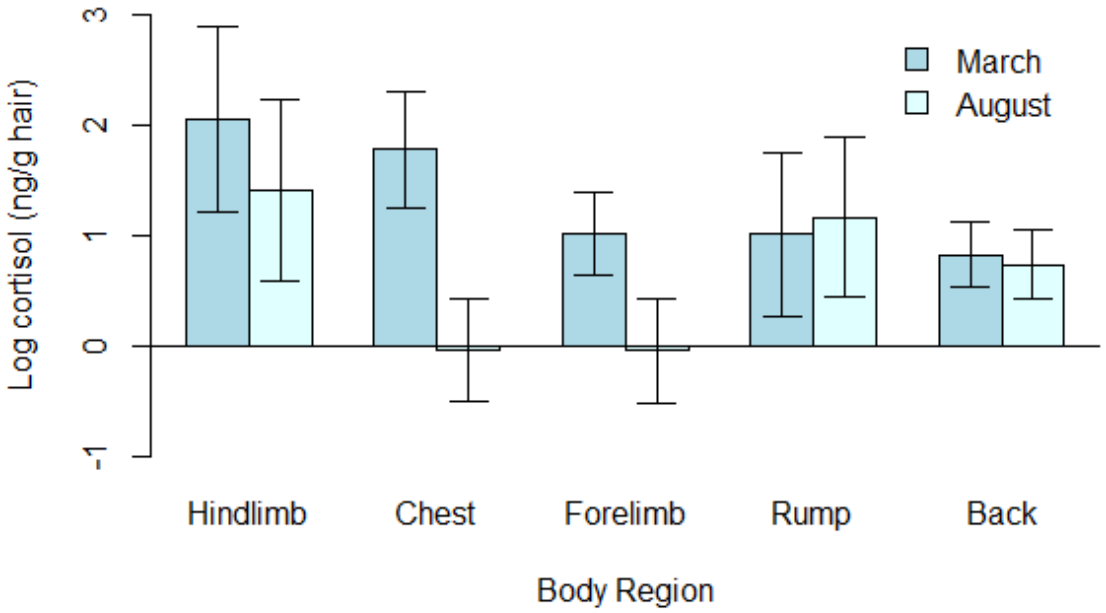

Figure 1.1 Mean hair cortisol concentration at different body regions in 8 captive Vancouver Island marmots during March and August. Hair cortisol concentration is given as the log-transformed concentration of cortisol (ng) per gram of dry hair. Error bars indicate 95% confidence interval. There is a main effect of sample month ($F = 17.572$; $df = 1, 63$; $p < 0.0001$) and body region ($F = 6.373$; $df = 4, 63$; $p = 0.000226$) and a significant interaction ($F = 4.573$; $df = 4, 63$; $p = 0.00263$). The chest is significantly different between seasons and the left hind limb is significantly different from the right forelimb, the rump and the back.

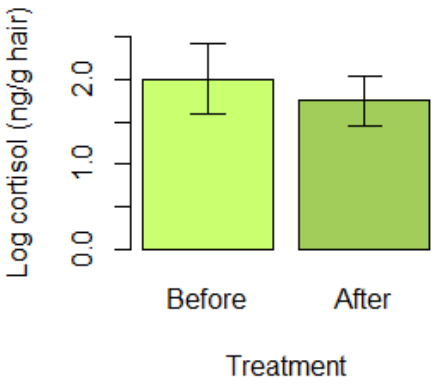

Figure 1.2 Mean hair cortisol concentration in mink pelts before and after a treatment with arsenic soap. Hair cortisol concentration is given as the log-transformed concentration of cortisol (ng) per gram of dry hair. Error bars indicate 95% confidence interval. There was no significant difference between treatments ($t = -1.5206$, $df = 10$, $p = 0.1593$).

Appendix A: Transformation of cortisol concentrations using the natural logarithm

The distribution of hair cortisol concentrations in Vancouver Island marmot (*M. vancouverensis*) and American mink (*N. vison*) was assessed for signs of normality and tested for normality using Pearson's chi-square test and Lilliefor's test. Hair cortisol concentration estimates were available from a total of 130 Vancouver Island marmots. Visual inspection of the data revealed that the distribution approximated a logarithmic distribution (see figure A.1) with marked asymmetry (kurtosis: 12.746), right skew (skewness: 2.8133) and a geometric mean (G) approximately equal to the median (m) (G/m = 1.0507). Pearson's chi-square test for normality and Lilliefor's test for normality both indicated a strong likelihood that the sample did not arise from a normally-distributed population ($p < 0.001$ in both cases).

Methods that have been reported in the literature suggest that cortisol concentrations in hair (Malcolm et al. 2013; Ashley et al. 2011), saliva (Chen et al. 2011; De Lange et al. 1997) and excreta (Bosson et al. 2009) are log-normally distributed within populations. As such, all data were transformed with the natural logarithm and reassessed for signs of normality. Visual inspection of the transformed data showed that the distribution approximated a normal distribution (see figure 1.1) with an increase in symmetry (kurtosis: 4.9201), slight left skew (skewness: -0.3365), an arithmetic mean ($\bar{x}$) approximately equal to the median (m) ($\bar{x}$/m = 1.0306) and a standard deviation of 0.9968. Pearson's chi-squared test for normality suggested that the sample was drawn from a log-normal distribution ($0.0902 < p < 0.181$). Lilliefor's test did not provide strong evidence for this ($p = 0.0261$), however visual inspection of a normal quantile-quantile plot shows data sufficiently approximate normality following transformation (see figure A.2).

Hair cortisol concentration estimates were available from a total of 11 American mink pelts. Visual inspection of the data revealed that the distribution approximated a logarithmic distribution

with marked asymmetry (kurtosis: 7.619), right skew (skewness: 2.175) and geometric mean (G) approximately equal to the median (m) ($\mathrm{G}/\mathrm{m} = 1.233$). A Pearson's chi-square test for normality indicated a strong likelihood that the samples were no drawn from a normal distribution ($p < 0.01$), as did Lilliefor's test ($p = 0.00357$). The data were transformed using the natural logarithm. The distribution of the transformed data more closely approximated a normal distribution with an increase in symmetry (kurtosis: 3.0511), a decrease in right skew (skewness: 0.0860) and an arithmetic mean ($\bar{x}$) approximately equal to the median (m) ($\bar{x}/\mathrm{m} = 1.125$). Pearson's chi-squared test for normality shows evidence that the transformed data are normally distributed ($0 < p < 0.122$) and Lilliefor's test showed no significant departure from normality ($p = 0.0768$).

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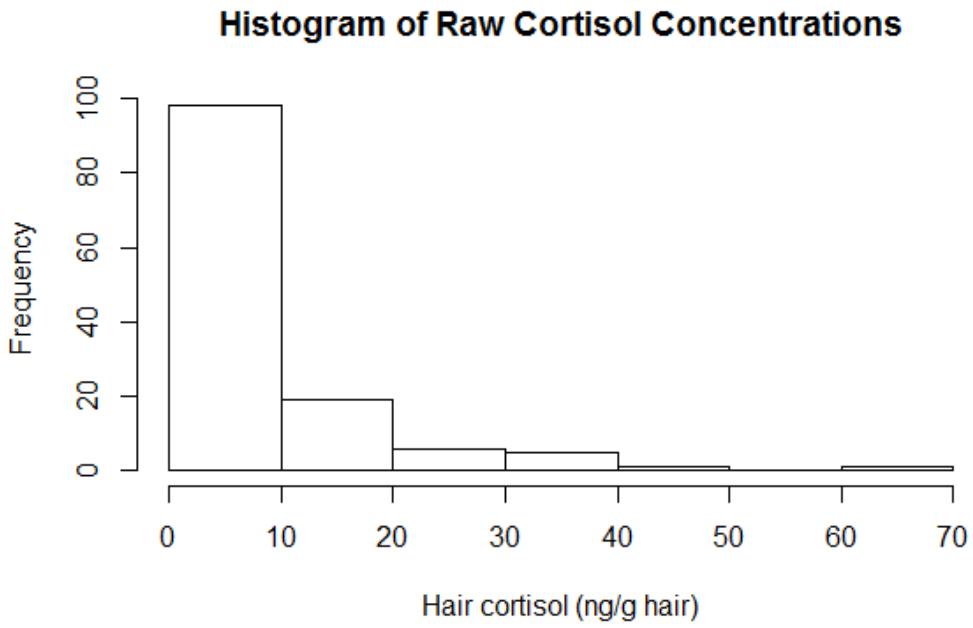

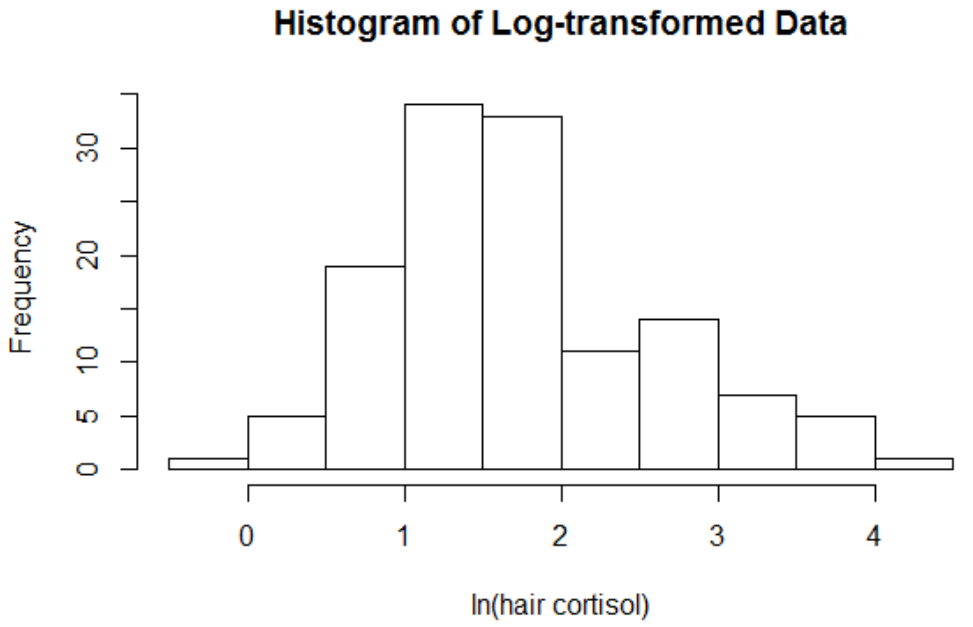

Figure A.1 The frequency of observed hair cortisol concentrations in 130 Vancouver Island marmots. Marmots living in different locations were pooled. Top: raw data. Bottom: data transformed using the natural logarithm.

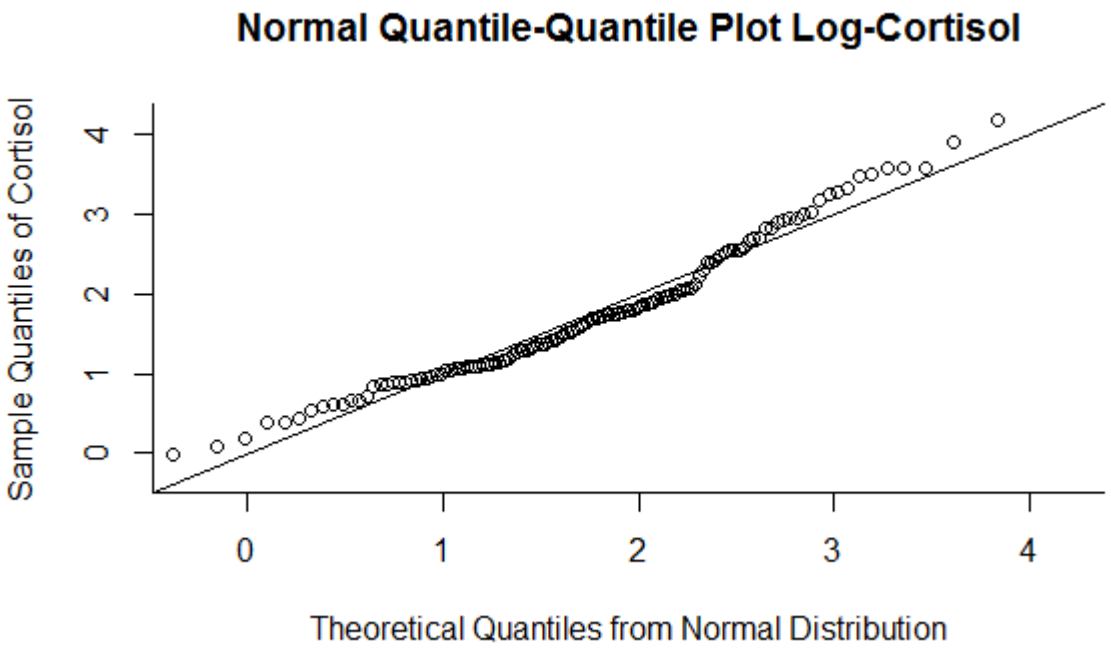

Figure A.2 The normal quantile-quantile plot of expected (normal) distribution and the observed distribution of cortisol concentration in 130 Vancouver Island marmots, following transformation with the natural logarithm.

Appendix B: Additional pelts used to test the effect of arsenical soap and a test for the effect of borax

I collected additional pelts to observe the effects of arsenical soap treatment and the effect of borax treatment on hair cortisol concentration in a variety of species. In addition to the remains of mink collected on fur farms, the remains of three Eastern red squirrels (*Tamiasciuris hudsonicus*), three red foxes (*Vulpes vulpes*) and three snowshoe hares (*Lepus americanus*) were treated with arsenic. The red squirrels were collected between 2013 and 2014 as part of a long-term small mammal study at Algonquin Provincial Park on Ontario, Canada. The red foxes were found dead on Laurentian University campus in Sudbury, Ontario, Canada and may have died as a result of motor vehicle collisions. The snowshoes hares were found on roadsides around Northeastern Ontario. To test the effects of borax, the remains of nine animals were collected from roadsides in Northeastern and Southwestern Ontario: three raccoons (*Procyon lotor*), three groundhogs (*Marmota monax*), two striped skunks (*Mephistis mephistis*) and one American mink (*Neovison vison*).

Study skins were prepared using the methods outlined above. The pelts used to test the effects of borax were treated as follows: one half-teaspoon (2.5 mL) of household borax was sprinkled onto the right side of the rump, first on the leather, then on the fur. The borax was worked into the skin for 10 seconds on each side using gloved fingertips. Each skins was then rinsed liberally with distilled water. All skins were left to dry overnight in the fume hood. Hair cortisol concentration was determined as described above except that the mixed species samples were analyzed on a common plate so the hair cortisol analysis protocol was amended to accommodate interspecies variation; extracts were concentrated 5 times instead of ten times.

Pelts were collected opportunistically, generally during the course of a roadkill surveys, so sample sizes for each species were very small. This precluded the use of statistical testing to determine if arsenic treatment or borax treatment caused a significant change in cortisol

concentration within each species. However, a similar trend can be observed among the mink pelts that were treated with arsenic and the additional pelts that received the same treatment (see figure B.1). Only eight of the nine pelts used in the borax treatment, representing three different species, yielded results along the standard curve. As such, a statistical test was not conducted with these samples either. It is possible that these data show a trend for a decrease in cortisol. However, the 95% confidence interval around the mean of the hair cortisol concentration before borax treatment overlaps considerably with the interval around the mean of hair cortisol concentration after treatment, if samples are pooled across species (see figure B.2). Therefore, it is difficult to draw a conclusion.

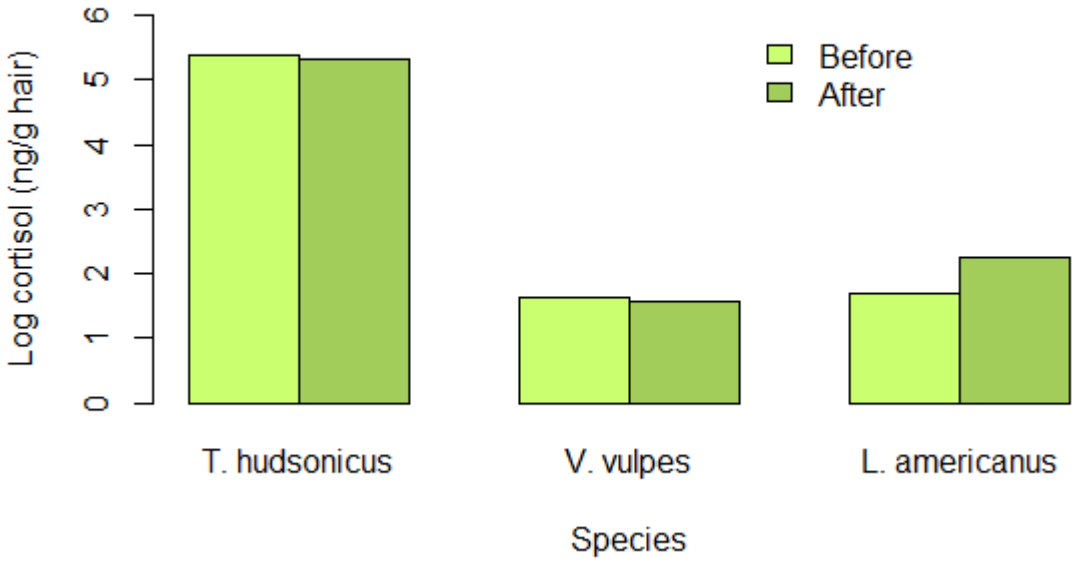

Figure B.1 Mean hair cortisol concentration in pelts of three species (*Tamiasciurus hudsonicus, Vulpes vulpes, Lepus americanus*) before and after a treatment with arsenic soap. Hair cortisol concentration is given as the log-transformed concentration of cortisol (ng) per gram of dry hair (n=3 for each group).

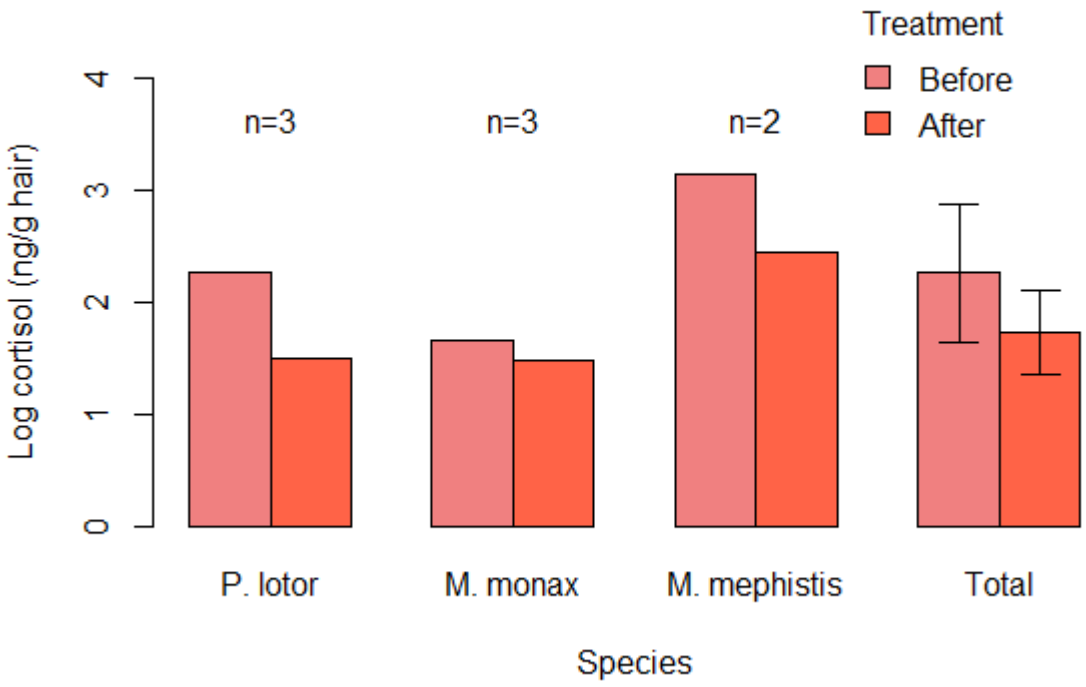

Figure B.2 Mean hair cortisol concentration in hair samples taken before and after treatment with borax. Hair cortisol concentration is given as the log-transformed concentration of cortisol (ng) per gram of dry hair. Error bars indicate 95% confidence interval. Sample sizes listed above.

CHAPTER TWO – Hair cortisol concentrations associated with the captive breeding and release of Vancouver Island marmots

Abstract

As part of the management and conservation of endangered species, a large proportion of the extant population may be exposed to the stressors of the captive environment or the stressors associated with transportation and release to a novel environment. This is particularly true of species involved in captive breeding and release programs. The Vancouver Island marmot is a critically endangered species. A breeding population is maintained in captivity and most of the offspring produced each year are released to the wild. Chronically-elevated cortisol levels could explain two phenomena that undermine success of the program: (1) breeding pairs have low success rates, despite food provisioning and other benefits of captivity and (2) captive-reared marmots have low over-winter survival during their first year after release from captivity. In this chapter, I used hair cortisol analysis to investigate differences among the wild-reared population, the captive-release population and the captive population. I found that captive female marmots have lower cortisol levels than both captive males and wild-reared females, suggesting that captivity does not contribute to high cortisol exposure and low reproductive success via the female within a breeding pair. I found that captive-reared marmots have significantly higher cortisol levels during their first year in the wild than wild-reared marmots or captive-reared marmots that have spent two or more years in the wild. Release from captivity is not likely to have long-term effects on marmots.

Introduction

Glucocorticoid hormones (GCs) are secreted by the adrenal gland to balance an animal's energetic demands (McEwen and Wingfield, 2003) which vary based on factors intrinsic to the animal (e.g. reproductive status) and extrinsic to the animal (e.g. predation risk) (Dantzer *et al.*, 2014). Activation of low-affinity GC receptors promotes gluconeogenesis so that high concentrations of GCs mobilize energy reserves (Herman *et al.*, 2016); this is an adaptive response when an animal is faced with a nutritional challenge or another physical threat to its survival (Reeder and Kramer, 2005). GCs levels that are chronically elevated may be indicative of poor living conditions in which considerable adrenal activity is necessary to survive. GC levels may also be elevated in response to a perceived threat to survival and may be driven by some "emotional" state (i.e. fear) (Reeder and Kramer, 2005). For these reasons, GC levels can be used as one measure of animal welfare in captive environments (Palme, 2012).

Experimental manipulations of GC levels have shown that increasing GCs can suppress immune function (Padgett and Glaser, 2003) and female fertility (Nepomnaschy *et al.*, 2007; Ralph *et al.*, 2016). Thus monitoring GC levels in captive animals may identify captive conditions that are both challenging to an animal's welfare and detrimental to its health and reproduction. Captive breeding is a conservation practice that releases animals from the pressures of their natural environment with the purpose of facilitating reproduction in a controlled setting (Lynch and O'Hely, 2001). But, in species that respond poorly to captivity, the captive environment may inadvertently subject individuals to novel pressures that are not conducive to breeding (Mason, 2010). An important criterion used to evaluate the success of captive breeding programs is the possibility of maintaining self-sustaining populations of reproductive adults in captivity (Kleiman, 1989; Snyder *et al.*, 1996). The captive populations of some endangered species, such as cheetahs, *Acinonyx jubatus* and pygmy rabbits, *Brachylagus idahoensis*, show higher-than-expected rates of reproductive failure and

high maternal GC levels, which has raised concerns about the feasibility of these programs (Marker and O'Brien, 1989; Scarlata *et al.*, 2012; Terio *et al.*, 1999; Wells *et al.*, 2004).

Offspring born in a captive breeding program must subsequently survive introduction to the wild. Release to a novel environment may expose individuals to a variety of other challenges that were absent from the captive environment (Teixeira *et al.*, 2007). Transportation and translocation has been associated with high GC levels in African elephants, *Loxodonta Africana*, and can persist for years after a population is translocated to a new wildlife refuge (Jachowski *et al.*, 2012). Release from captivity leads to an increase in GC levels in Persian fallow deer, *Dama mesopotamica*, which is related to a decline in survival (Zidon *et al.*, 2009). It is not immediately clear what elevated GCs indicate in a newly-released population, but they may be part of the acclimation response. However, consistently elevated GC levels following an acclimation period may be indicative of a mismatch between the needs of a captive-reared species and its native habitat.

The Vancouver Island marmot is a critically endangered species of ground squirrel that is dependent on a captive breeding program for its continued survival in the wild (Roach, 2017). In captivity, marmots are food provisioned, quarantined and protected from predation and adverse weather conditions (McAdie, 2004). In addition, marmots are housed in breeding pairs and adults of the same sex rarely interact thus curtailing intrasexual aggression, which occurs in the wild (Brashares *et al.*, 2010; Heard, 1977). Captive marmots appear to experience a less challenging life than free-ranging conspecifics. Nonetheless, breeding pairs are no more successful in captivity than they are in the wild where approximately 40% of pairs mate successfully (Bryant, 2005; Jackson *et al.*, 2015). In many cases, copulation or signs of copulation are recorded by zookeepers, but a breeding pair will not produce any young (Keeley *et al.*, 2012). There may be some challenge in the captive environment that elicits an adrenal response and counteracts reproduction. Such a conclusion would

be based upon evidence that reproduction is suppressed when GC levels are high and that GC levels are elevated in captive individuals. In some marmot species, females may be able to suppress each other's reproduction through agonistic encounters (Armitage, 2014; Wasser and Barash, 1983) and in the Alpine marmot, *Marmota marmota,* high GC levels are related to poor reproduction (Hackländer *et al.*, 2003). It is possible that elevated GC levels interact with the reproductive system of female marmots to prevent pregnancy. By contrast, the relationship between GC levels and male fertility in marmots is less clear. In *M marmota,* the direction of the relationship between male fertility (testosterone levels) and GCs are dependent on a male's social status and age (Arnold and Dittami, 1997). Thus baseline GC levels alone are poor predictors of reproductive capacity in males. In addition, the potential threats that an animal may perceive in a captive environment are varied, but include such things as close proximity to humans, an artificial light-dark cycle and constraints on natural behaviours (Morgan and Tromborg, 2007). These threats can elicit an adrenal response and may do so in a sex-specific manner. Sex differences in baseline GCs of captive animals have been reported in several species (He *et al.*, 2014; Narayan *et al.*, 2010; Wielebnowski *et al.*, 2002). If there is something in the captive environment to which female marmots are averse, captive females may have elevated GC levels which could prevent them from becoming pregnant despite being in good condition and displaying normal sexual behaviour.

Research has shown that marmots that are released from captivity experience higher mortality rates than their wild-reared counterparts (Aaltonen *et al.*, 2009; Jackson *et al.*, 2016). Wild-reared marmots rarely die during hibernation (Bryant and Page, 2005), but a recent study found that over-winter mortality was significant among newly-released marmots (i.e., those that were hibernating in the wild for their first time) (Jackson *et al.*, 2016). Vancouver Island marmots are efficient hibernators under normal conditions (Bryant and McAdie, 2003), so the failure of captive-release marmots to survive their first winter could be the result of an aversive response to the novel

environment (Jackson *et al.*, 2016). If so, newly-released animals would have higher GC levels than their wild-reared counterparts However, established marmots (that have spent one winter in the wild already) survive hibernation as well as their wild-reared counterparts (Jackson *et al.*, 2016). This would suggest that marmots acclimate to Vancouver Island after more than one year in the wild and become more capable of overcoming the challenges of the natural environment. If so, established animals would have similar GC levels to their wild-reared counterparts. I will measure GC levels among the captive-release animals to investigate a possible relationship between release from captivity and adrenal activity by contrasting newly-released animals, with those that have become established in the wild and their wild-reared counterparts.

GCs are closely related to reproduction and social behaviour in many species (Dantzer *et al.*, 2014). As such, it may differ between males and females and it may change over the course of development. This study is the first to quantify GCs in the hair of Vancouver Island marmots, so the effect of age and sex on hair cortisol concentration is not yet known. In order to interpret hair cortisol concentration as an indicator of chronic stress, I described the variability in hair cortisol due to two intrinsic factors: age and sex. I expected that age would relate to hair cortisol concentration, as previous studies have shown that there is a relationship between hair cortisol and age in other species (Fourie *et al.*, 2015; González-de-la-Vara *et al.*, 2011). I expected that sex would relate to hair cortisol concentration as I expected that female marmots are susceptible to challenges in the captive environment which undermine their reproductive success. I also determined if extrinsic factors were related to hair cortisol concentration. I compared GC levels in captive marmots and free-ranging animals. I predicted that GC levels in captive and free-ranging females would not be significantly different as their reproductive success is similar. However, I expected male marmots to have lower GC levels in captivity than in the wild because they are protected from agonistic encounters, predators and other challenges in the natural environment. Finally, given the poor survival of newly-

released marmots and the normal survival rates among established marmots, I expected the newly-released group to have high GC levels compared to wild-reared animals and I expected the established group to have the same GC levels as wild-reared animals.

Methods

Study animals

Captive Vancouver Island marmots were housed at the Toronto Zoo or the Calgary Zoo in designated breeding facilities that are not accessible to the public. With the exception of one yearling marmot at the Toronto Zoo, all adult marmots were housed in breeding pairs and young of the year were housed with their parents. All marmots were sampled after completing their moult in July. Marmots at the Toronto Zoo were sampled in late August when they were anaesthetized for routine veterinary care using isofluorane gas. This procedure was approved by the Toronto Zoo's Animal Care and Research Committee (REF No: 2016-03-01). Marmots at the Calgary Zoo were sampled in December during hibernation and anaesthetic was not necessary.

The remaining marmots were sampled on Vancouver Island. Some were sampled at the Tony Barrett Marmot Recovery Centre prior to their release in active colonies and others were lived trapped following methods described by Bryant (1996). Marmots that were held at the Tony Barrett facility were only housed for short periods of time. Generally, these marmots were recovering from a minor surgery following implantation of a radio-transmitter but may otherwise have been acclimating to the high altitude environment prior to their release from captivity (McAdie, 2004). Marmots that were live-trapped live in colonies on Vancouver Island and are regularly handled by the Marmot Recovery Team for veterinary care, radio-telemetry or translocation.

Sample collection

Samples were drawn consistently from the backs of captive marmots (upper back, near the shoulders) and from the backs of free-ranging marmots (dorsal side, not further specified). To sample captive marmots, a 2 cm x 2 cm patch of fur was shaved from the back of each animal using clippers to cut as close to the skin as possible. Clippers were cleaned using 70% isopropanol and compressed air between samples. Free-ranging marmots were sampled opportunistically by the Marmot Recovery Team. Field technicians or veterinary personnel plucked or clipped a small tuft of hair (minimum 75 hairs) from the dorsum of physically restrained animals without the use of anaesthetic or by collecting hair when animals are anaesthetized for other veterinary care (M. McAdie, pers. comm.).

Marmots moult during the month of July (Naughton, 2014) so hair cortisol concentrations are likely to reflect the circulating cortisol concentrations during this period of hair growth (Kirschbaum *et al.*, 2009). As such, marmots were classified as "captive" if they had been living in captivity during their most recent moult and "release" if they had moulted at least once in the wild since being released, regardless of their living conditions at the time of sampling. If marmots were sampled between the 1st and 21st of July, these samples were considered to be representative of the preceding year, unless the marmot was an adult male (> 2 years of age). Adult males undergo the moult first (Naughton, 2014), and would therefore have a coat of new fur by mid-July; all samples of adult males taken during that time period would be representative of the contemporary year. All samples were collected in plain white envelopes or yellow coin envelopes and stored at room temperature until use.

Hair cortisol analysis

Cortisol was extracted in methanol and quantified using enzymeimmunoassay as described in the first chapter of this thesis. Inter-assay CVs were calculated by running external controls at 25% and 65% binding in duplicate on each plate. The CV for high control (25% binding) was 5.7% and for low control (65% binding) was 5.5%. Along with monitoring the CV of each duplicate, intra-assay CVs were further evaluated by loading a pooled fecal extract diluted to 50% binding repeatedly across the plate. For this assay, the intra-assay CV was 3.6%.

Statistical analyses

All cortisol concentrations were natural logarithm transformed to meet assumptions of normality. See Appendix A of this thesis for further information. A linear regression model was tested to determine if there was a relationship between age and cortisol concentration. A two-way analysis of variance (ANOVA) was used to determine if there was an effect of sex or captivity on hair cortisol concentration. Tukey's Honestly Significant Difference (HSD) was used to determine if means were significantly different in four pairwise comparisons: (1) male and females in captivity, (2) males and females in the wild, (3) females in captivity and in the wild, (4) males in captivity and in the wild. One-way ANOVA was used to determine the difference in hair cortisol concentration within the wild environment, between individuals born in the wild, newly-released captive-reared individuals and captive-reared animals that had become established in the wild. Tukey's HSD was used to determine if means were significantly different between groups. All analyses were done using R statistical software (R Core Team 2015).

Results

A total of 49 wild-reared marmots were sampled between 2007 and 2016 from 12 different colonies on the island. The sample included 24 males and 25 females and ranged in age from less than one

year to 10 years at the time of sampling. There was no significant relationship between age and hair cortisol concentration (adjusted R^2 = - 0.01453; F = 0.3123; df = 1, 47; p = 0.5789; data not shown).

A total of 25 captive animals were sampled of which 8 were housed at the Toronto Zoo and 17 were housed at the Calgary Zoo. Eleven captive marmots were female and 14 were male. Both captivity and sex had a significant effect on hair cortisol concentration (captivity: F = 15.783; df = 1, 73; p = 0.000165; sex: F = 4.885; df = 1, 73; p = 0.03023). The interaction term was not significant (F = 0.037; df = 1, 72; p = 0.848). The mean cortisol level in captive females was 0.946 (95% confidence interval: 0.863, 1.028) and in captive males was 1.337 (95% CI: 1.201, 1.473). The mean cortisol level in wild females was 1.673 (95% CI: 1.511, 1.834) and in wild males was 1.999 (95% CI: 1.824, 2.175). Tukey's HSD for pairwise comparisons revealed that there was a significant difference between wild females and captive females (HSD: 0.727, p = 0.0214) and a significant difference between wild males and captive males (HSD: 0.622, p = 0.0259), but differences between the sexes in either environment were not significant (see figure 2.1).

A total of 16 captive-release animals were sampled. Nine animals were newly-released. The mean number of days newly-released marmots spent in the wild was 367 ± 4 (standard error of the mean). Seven animals were established. The mean number of days established marmots spent in the wild was 1553 ± 68 (standard error of the mean). No sex difference was detected in wild marmots (figure 2.1), so males and females were pooled for this analysis. There was a statistically significant difference between hair cortisol concentration in newly-released, established and wild-reared marmots (F = 4.231; df = 2, 62; p = 0.0190). In newly-released marmots, the mean cortisol level was 2.372 (95% confidence interval: 2.084, 2.660); in established marmots, the mean cortisol level was 1.149 (95% CI: 0.971, 1.327); in wild-reared marmots, the mean cortisol level was 1.840 (95% CI:

1.651, 2.028). Tukey's HSD for pairwise comparisons revealed that there was a significant difference between newly-released animals and established animals (HSD: 1.223, p = 0.0137; see figure 2.2).

Discussion

These results showed no relationship between age and hair cortisol concentration. Age was found to be a significant predictor of hair cortisol in a study of dairy cattle (González-de-la-Vara *et al.*, 2011) and baboons (Fourie *et al.*, 2015). Both studies found higher hair cortisol concentration within juveniles compared to adults. This study involved 30 juveniles (0-2 years of age) and 19 marmots of reproductive age (3 years of age and older, (Keeley *et al.*, 2012)); if a similar relationship exists in marmots, I should have been able to detect it using this data set. It seems likely that this species does not experience marked changes in baseline cortisol at different life stages. These results also demonstrated that there was no sex difference in hair cortisol concentrations when males and females were compared within the same environment. Sex differences in hair cortisol concentration have not been found in two species of free-ranging bears (Bechshøft *et al.*, 2013; Macbeth *et al.*, 2010) or Canada lynx (Terwissen *et al.*, 2013), nor in populations of captive ungulates (Ashley *et al.*, 2011), Asiatic bears (Malcolm *et al.*, 2013) or domestic dogs (Bennett and Hayssen, 2010). However, many of these studies did not investigate if sex differences vary between different living conditions (but see Malcolm *et al.*, 2013). I expected that males and females would have different cortisol levels in captivity, but that wild marmots would have similar cortisol levels irrespective of sex. These results show that the cortisol levels in captive females are not significantly different from captive males. Therefore I find no evidence that captivity is particularly challenging for female marmots compared to their male mates.

I had expected to see a higher level of cortisol in female marmots that could account for the reproductive suppression that seems to occur in the captive population. However, I found lower

cortisol levels in captive animals than in wild animals. This could be because wild females had fewer energetic reserves at the time of sampling than captive females, owing to the demands of gestation and lactation. I have assumed that hair cortisol concentration is reflective of cortisol in circulation during the month of July when hair is growing (Naughton, 2014); at that time, parturition would have occurred and female marmots would have weaned the litter of the year (Keeley *et al.*, 2012). It is possible that the high cortisol levels reflected an energy deficit in free-ranging animals, while captive females had lower cortisol because they had access to more food during the reproductive season. However, female marmots who have weaned a litter of pups are generally the last to moult, presumably because they are rebuilding their energy stores (Naughton, 2014). This would suggest that female marmots do not grow hair during periods of nutritional stress that form part of their normal life history. Given that hair cortisol analysis measures cortisol levels that correspond to systemic cortisol during the period of hair growth, it is highly unlikely that cortisol levels associated with the immediate demands of reproduction could be measured in this species.

Very low baseline cortisol levels could also be a result of adrenal exhaustion which occurs when the adrenal glands are chronically overstimulated (Herman *et al.*, 2016). One would expect low levels of cortisol in animals with adrenal exhaustion, but would also expect animals to be in poor health (Herman *et al.*, 2016). However, captive marmots have a normal lifespan in captivity suggesting that they do not have health issues that may be associated with adrenal exhaustion. Furthermore, animals that are released from captivity show initially high levels of cortisol, suggesting that the adrenal gland is responsive in captive-reared animals and will upregulate GC signalling in the face of novel pressures. Therefore it is unlikely that female, captive marmots experience excessive cortisol exposure. Low reproductive rates may be unrelated to cortisol entirely but could be due to other factors such as inbreeding effects on sperm quality (Asa *et al.*, 2007; Gage *et al.*, 2006; van

Eldik *et al.*, 2006). Further research is necessary to understand the reasons for reproductive success in this species.

Newly-released marmots have significantly higher cortisol levels than established marmots. These results parallel the results found by Jackson and colleagues (2016) when they examined over-winter mortality rates among marmots. They found that newly-released marmots survived poorly compared to wild-reared or "established" marmots that had already survived for a year in the wild. Therefore it is possible that this elevated cortisol level is associated with poor survival. The observation of high cortisol levels points to two possible mechanisms for poor first-winter survival that should be further investigated. It is possible that newly-release marmots are learning to forage in the wild and have lower caloric intake than wild-born individuals or established marmots. The captive diet differs from a wild diet and marmots do not have to forage in a captive environment (McAdie, 2004). Captive-reared animals are often reported to forage ineffectively following their release to the wild, as they may have to learn how to locate good quality food sources or compete with other species to access food (Horwich, 1989; Peignot *et al.*, 2008; Stoinski and Beck, 2004). Difficulty in accessing food resources could explain in part the high mortality rates observed in newly-released marmots. GC levels are correlated with energy reserves in many mammals such that high GC levels are indicative of poor body condition (Cattet *et al.*, 2014; Hing *et al.*, 2017; Kershaw *et al.*, 2017) and a decrease in food intake (Bechshøft *et al.*, 2013; Ortiz *et al.*, 2001; Pride, 2005). If captive-release marmots are poor foragers compared to their wild-born counterparts, then their GC levels would reflect this.

However, captive-release marmots suffer from higher mortality rates when they are released later in the season (Jackson *et al.*, 2016). This suggests that poor survival is not due to low caloric intake, because those that are released later in the season spend longer in the captive, food-

provisioned environment. It may instead mean that early-release marmots have a longer time to adjust to the new environment, and have acclimated by the time they enter hibernation while the late-release marmots may still be experiencing elevated cortisol at this critical time (Jackson *et al.*, 2016). The technique used in this study does not allow for differentiating between cortisol levels during different seasons, therefore it is difficult to determine how high cortisol levels are immediately preceding hibernation. It is also not clear how high GC levels are related to hibernation success in the absence of a nutritional deficit. It is possible that high GC levels due to the challenges of release from captivity have an immune suppressive effect (Dhabhar, 2000). Marmots are susceptible to pathogens such as *Yersinia entercolitica, Escherichia coli* and *Mycoplasma* sp. (McAdie, 2004). In one of the instances of over-winter mortality recorded among wild-reared marmots, bacterial infection was determined to be the most likely cause of death (Bryant *et al.*, 2002). Therefore it is possible that captive-reared marmots could succumb to disease during hibernation. Further research will be necessary to understand the immune capacity of captive-reared marmots and released marmots.

These results would also benefit considerably from experimental studies in a closely related species such as the groundhog *Marmota monax,* or another species of ground squirrel that is not critically endangered. The functional cost of high GC levels are not known in this species. Studies of *M marmota* have shown a correlation between high cortisol levels and low reproductive success of females but have shown a more complex relationship between fertility and cortisol levels in males that is dependent on social status (Arnold and Dittami, 1997; Hackländer *et al.*, 2003). Similarly, Costantini *et al.*, (2012)showed a relationship between cortisol and plasma antioxidant capacity in *M marmota*, but it was mediated by personality type. Social status and personality are both important factors to consider in understanding the role of GCs in a social species such as the Vancouver Island marmot. This is complicated by husbandry practices that preclude the organization of social

structures among captive animals. If further research is to be undertaken involving marmot biology, it may be advisable to consider allowing captive animals to live in normal family groups. Given that the captive breeding and release program must continue into the immediate future to ensure the species persistence (Jackson *et al.*, 2015; Roach, 2017), it is important to consider how research can support this goal and what research questions can feasibly be addressed. It is encouraging to the efforts of the Marmot Recovery Team that the practices of keeping marmots in captivity and releasing wild-reared animals to the wild have not created subpopulations of animals with chronically elevated cortisol levels. But this is only the case if an important assumption, that Vancouver Island marmots with low cortisol levels live in better conditions than those with high cortisol levels, holds true. An analysis of the role of cortisol in this species is necessary in order to fully understand the results presented here.

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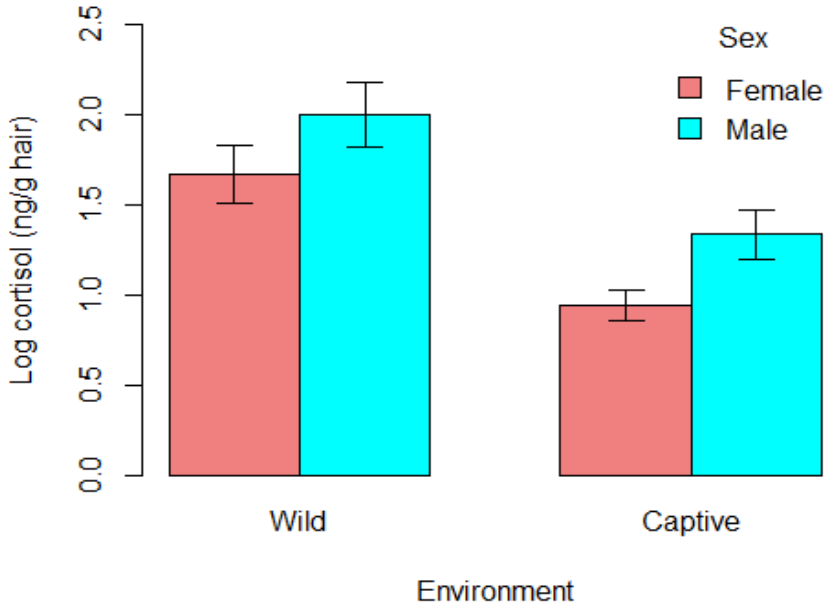

Figure 2.1 Mean hair cortisol concentration in captive and wild Vancouver Island marmots. Error bars give the 95% confidence interval about the mean. Hair cortisol concentration is given as log-transformed concentration of cortisol (ng) per gram of dried hair. The main effect of sex is significant ($F = 4.885$; $df = 1, 73$; $p = 0.03023$) and the main effect of environment is significant ($F = 15.783$; $df = 1, 73$; $p = 0.000165$), with no significant interaction. Females in captivity have significantly lower cortisol levels than males in captivity, and captive animals have significantly lower cortisol levels than wild animals. There is no significant difference between males and females in the wild.

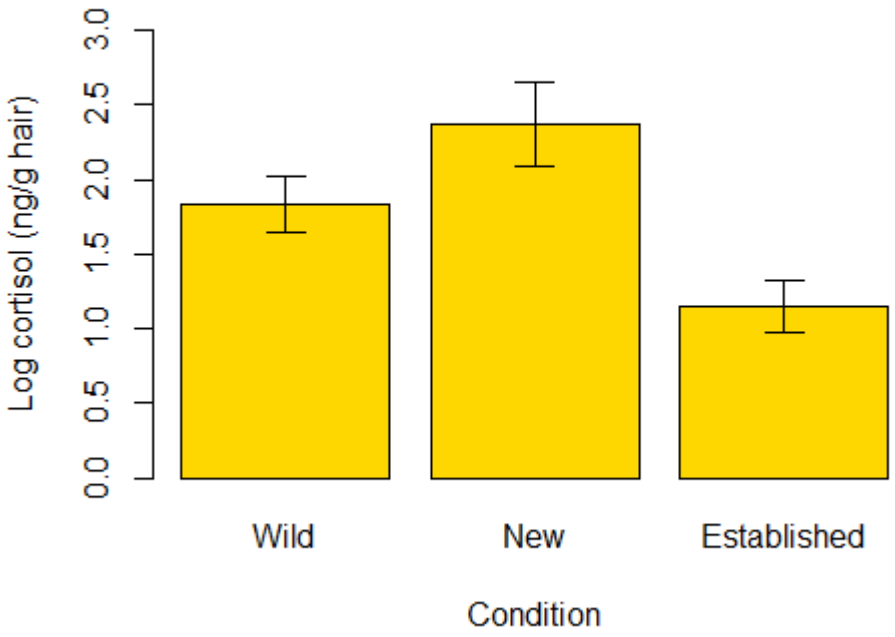

Figure 2.2 Mean hair cortisol concentration in wild-reared marmots, newly-released captive-reared marmots and marmots that were released from captivity for more than one year. Error bars indicate 95% confidence interval. Hair cortisol concentration is given as the log-transformed concentration of cortisol (ng) per gram of dried hair. There is a statistically significant difference between groups ($F = 4.231$; $df = 2, 62$; $p = 0.0190$). Newly established marmots have higher hair cortisol concentrations than those that have become established in the wild for more than one year.

Appendix C: Reproductive indices in captive marmots 2005-2016

The Vancouver Island Marmot Recovery Team provided reproductive indices in the captive population between 2005 and 2015 as an appendix in their 2015 report. It showed that mean litter size was 3.39 ± 0.43, well within the range Bryant reported in 2005 (3.38 ± 1.14). The proportion of females mating reported by the Recovery Team was the same as the one reported by Bryant among wild marmots (0.41). Only the sex ratio of offspring at birth and the age of first reproduction had not been compared to Bryant's results from 2005. In the interest of fully updating the reproductive indices for this critically endangered species, I have reported the age of first reproduction and the sex ratio of offspring here, and tested these for a significant departure from Bryant (2005).

The age of first reproduction was determined using the birthdate and first recorded offspring produced by captive females. If a marmot entered the records as an adult (>2 years) and previous reproductive success could not be determined using the zoo records, this individual was not counted. A one-way ANOVA was used to determine if the mean age of first reproduction is different at either captive facility or in the wild as reported by Bryant (2005). Between 2005 and 2016, 19 marmots gave birth for their first time: eight at the Toronto Zoo and 11 at the Calgary Zoo. The mean age of first reproduction at the Toronto Zoo was 3.75 years with a standard deviation of 1.67. The mean age of first reproduction at the Calgary Zoo was 3.82 years with a standard deviation of 1.25. There was no difference between the age of first reproduction in either captive environment and in the wild as reported by Bryant (2005) ($F = 0.141$; $df = 2, 34$; $p = 0.869$).

The offspring sex ratio was determined by counting all the offspring produced by each mated pair in captivity each year. Offspring whose sex could not be identified were counted as 'unknown' and were not used to determine the sex ratio. This ratio represents the sex ratio at birth rather than at weaning as offspring that died shortly after birth were included. A chi-squared test was

used to determine if the sex ratio of marmots born in captivity differed significantly from 1:1 at either facility. Between 2005 and 2016, 105 marmots were born in the Toronto Zoo and 124 marmots were born in the Calgary Zoo for a total of 229 individuals. At the Toronto Zoo, 47 offspring were female, 57 were male, and one offspring was not identified as male or female. This gives a proportion of 0.45 female and 0.55 male. At the Calgary Zoo, 50 offspring were female, 68 were male, and six were not identified as male or female. This gives a proportion of 0.42 female and 0.58 male. The ratio reported by Bryant (2005) was 1.04 females per male born in the wild for a proportion of 0.52 female and 0.48 male. There is no significant difference between the proportion reported by Bryant in the wild population and the proportions recorded in the captive population at either facility ($\chi^2 = 1.706$; df = 2, p = 0.426; See figure C.1).

Table C.2 Reproductive indices in captive and wild populations of Vancouver Island marmots. Data from 2005 published by Bryant. Data from 2015-6 published by Jackson et al. (2015) and this study (bold text). Asterisk indicates a statistically significant difference between the captive and wild population.

	Wild, 2005	Captive, 2005	Captive, 2015-6
Mean litter size ± standard deviation	3.38 ± 1.14	3.00 ± 1.44	3.39 ± 0.43
Proportion of females mating	0.41	0.38	0.41
Offspring sex ratio (females/males)	1.04	0.56*	0.78
Age at first reproduction	3.6	4.3	3.8

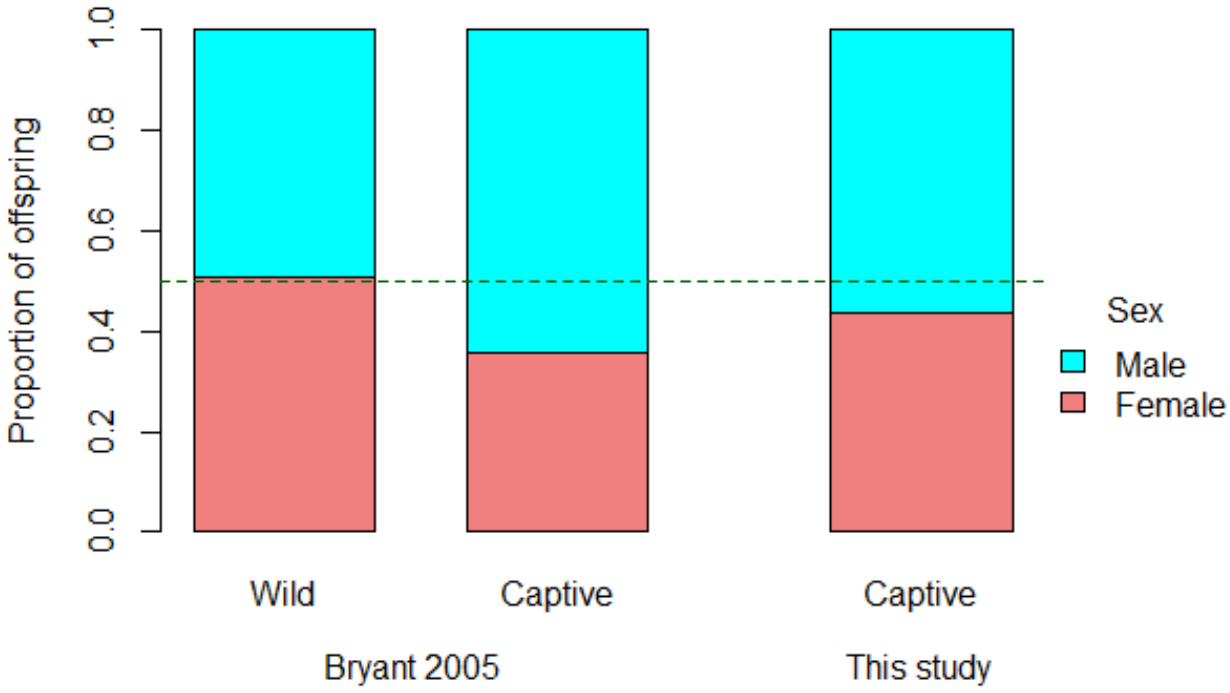

Figure C.1 The sex ratio of marmot offspring produced by the captive and wild populations between 1980 and 2004 (Bryant 2005) and by the captive populations at the Toronto and Calgary Zoos between 2005 and 2015. Dashed line indicates the expected ratio of 1:1. Bryant found that the sex ratio in captivity was significantly different from the wild, but this study found that the sex ratio of captive offspring was not different from the sex ratio Bryant reported in the wild.

CHAPTER THREE – A comparison of historic and contemporary cortisol levels in an endangered species that may be susceptible to human disturbance

Abstract

Many species at risk of extinction are threatened by broad anthropogenic changes to ecosystems and landscapes. But it can be difficult to assess potential threats because they may be ubiquitous across a species range. As such, no extant population can be used to determine physiological baselines that characterize populations living in suitable habitat. In many species, GCs increase in response to human activity and high GC levels may be a signal of low environmental quality in which animals must contend with many challenges. It is possible to measure glucocorticoid hormones (GCs) in historic hair samples using hair cortisol analysis which could be used to overcome the problem of ubiquitous disturbance, if historic populations predate the onset of the disturbance. The Vancouver Island marmot (*Marmota vancouverensis*) is a species of ground squirrel that is currently listed as critically endangered. A few hundred adults live on Vancouver Island but populations are not stable and this could be due to human disturbance such as high-altitude logging. Study skins were collected between 1910 and 1930, prior to the onset of most human activity in the subalpine region, and prior to the species decline. These were sampled to establish a historic baseline GC level for the species. Free-ranging marmots from 14 contemporary colonies (2008-2016) were sampled and analysis of variance showed significantly elevated GC levels at three colonies. These populations may be living in poor quality habitat. Generalized linear mixed models were tested using proximity to sources of human disturbance, summertime temperatures and marmot abundance as predictors of individual GC level. The most highly supported model showed a negative relationship between proximity of a colony to a logging road and GCs in marmots at that colony. Vancouver Island marmot colonies might be threatened by the creation and maintenance of linear features in the landscape.

Introduction

Human activity is implicated in severe biodiversity loss which may amount to the planet's sixth mass extinction event (Barnosky *et al.*, 2011; Dirzo *et al.*, 2014). In Canada, all species-at-risk are threatened to some degree by human activities including residential and commercial development; human intrusion and disturbance; and the modification of natural systems (McCune *et al.*, 2013). Many species will avoid human disturbance (Bautista *et al.*, 2004; George and Crooks, 2006) and populations can be extirpated from habitats that are highly disturbed or degraded due to human activity (Brodie *et al.*, 2014; Sawyer *et al.*, 2006). Other species persist despite human disturbance but may suffer some fitness consequences such as poor immune function or low reproductive output in disturbed habitats (Ellenberg *et al.*, 2007; French *et al.*, 2011, 2010). This suggests that there are human intolerant species for which anthropogenic modification to the landscape is a cause of habitat degradation. Humans may contribute to poor quality habitat if they become numerous in a landscape where native fauna perceive them as a constant threat (e.g., as a predator (Beale and Monaghan, 2004; Thiel *et al.*, 2008)), or if anthropogenic modification to a habitat makes an individual more susceptible to other noxious stimuli in the environment (e.g., removal of vegetation leaves animals with a lack of appropriate shelter (Seltmann *et al.*, 2017)).

Animals that are faced with challenges to overcome in their environment may upregulate activity of the adrenal gland, an endocrine gland that is involved in regulating energy use and osmotic balance (Herman *et al.*, 2016). Under normal circumstances, this will result in higher baseline levels of glucocorticoid hormones (GCs) (Dantzer *et al.*, 2014). Research has shown that human disturbance is related to an increase in baseline GC levels in populations of bird, mammals, reptiles and amphibians relative to conspecifics in areas of low disturbance (Dantzer *et al.*, 2014). The use of GCs to measure physiological disturbance and to determine populations of animals that are living with disturbance are basic goals in the field of conservation physiology (Wikelski and Cooke, 2006).

In species which are distributed in populations that experience variable amounts of disturbance, conservation physiology is a useful approach. But any critically endangered species which has been nearly extirpated from the wild likely exists in populations that are all subjected to disturbance; when studying these species, an appropriate reference population is lacking and inference about human disturbance and habitat quality may not be possible. In order for conservation physiology to be useful in such circumstances, it is necessary to approximate the GC levels of a population that does not experience anthropogenic disturbance. This may be possible by using data from historic populations that lived in a time period prior to the proliferation of the disturbance type (Bocharova *et al.*, 2013; Powell and Powell, 1986).

The Vancouver Island marmot (*Marmota vancouverensis*) is a critically endangered species of ground squirrel. It is a social species, living in small family groups or colonies in subalpine meadows on Vancouver Island, Canada (Heard, 1977). In the early 2000s, the population comprised fewer than 40 individuals (McAdie, 2004). Today, the species is dependent on a captive breeding and release program and intensive species management by the Marmot Recovery Team (Jackson *et al.*, 2015; Roach, 2017; Vancouver Island Marmot Recovery Team, 2008). Decisions about where to reintroduce and translocate animals are made by the recovery team based on numerous criteria for suitable habitat such as food availability and soil depth (Jackson *et al.*, 2015; Vancouver Island Marmot Recovery Team, 2008). However, researchers and wildlife managers risk overlooking some component of the habitat that mediates its suitability for marmots, thus establishing colonies in habitats that are suboptimal, because it is difficult to imagine all of a marmots needs. Measuring GC levels in different habitats may help us overcome this challenge by measuring marmot physiology directly and determining which habitats support marmots with greater adrenal activity, and therefore marmots that may be surviving in relatively worse environmental conditions than their conspecifics.

Vancouver Island marmots were not studied *in situ* until the 1970s when infrastructure built by the logging industry made their remote mountain habitats accessible (Bryant and Janz, 1996; Heard, 1977). Thus marmots have been living with human disturbance since their ecology was first described. However, Vancouver Island marmots were named and described in the early 1900s, when individuals from several colonies were shot and their pelts were sent to a natural history collection in California (Swarth, 1912). It is possible to use these pelts to measure historic GC levels using hair cortisol analysis. The use of hair as a substrate to measure systemic GC exposure is similar to other integrative measures such as urine (Sauve *et al.*, 2007), however there are several advantages to using hair over traditional substrates as GCs in hair reflect exposure during the weeks or months when hair is growing (i.e., during moulting season) (Kirschbaum *et al.*, 2009; Koren *et al.*, 2002; Malcolm *et al.*, 2013). Second, hair and molecules found within the hair shaft remain stable over extended periods of time, from several decades to centuries[1] (Bocharova *et al.*, 2013; Lewin *et al.*, 1982; Macbeth *et al.*, 2010; Webb *et al.*, 2010). [2]Therefore, hair samples from a natural history collection can be used to estimate chronic GC exposure in a population that lived one hundred years before present.

Two important assumptions underlie the use of historic populations to provide a baseline estimate of GC exposure in a species that is currently at risk of extinction, possibly due to human disturbance. First, historic populations lived during a period of little or no human disturbance. Indigenous people have lived on Vancouver Island for several thousand years and they practiced timber harvest and forest management in pre-contact times (Mackie, 2000). But these practices did not involve the use of chainsaws and motorized vehicles which began in the 1930s and 1940s, respectively (Rajala, 1998). Furthermore, high altitude timber harvest did not begin until the 1950s, having been previously confined to valley bottoms (Aaltonen *et al.*, 2009; Bryant and Janz, 1996). Today, British Columbia's timber harvest industry continues to have manifest impact on the

landscape: trees are removed from cutblocks to create deforested patches, logging roads are built along valleys and onto mountain sides (Bryant, 1998), and timber harvest has supplanted forest fire as a means of interrupting forest succession on Vancouver Island (Hebda et al. 2004). In addition, public roads have been constructed across Vancouver Island and, in the past 60 years, have been surfaced with asphalt, upgraded and widened to serve an ever-growing population of motorists (British Columbia Ministry of Transportation and Highways, 2012).; provincial highway 1A, for instance, was paved in 1967 as part of the Trans-Canada highway (MacLeod, 2014) and provincial highway 4 was paved in 1972 (British Columbia Ministry of Transportation and Highways, 2012)[3].

The second assumption is that historic populations were not already in decline when they were sampled. Using historic records of marmots, Bryant and Janz (1996) concluded that Vancouver Island marmot populations were thriving prior to 1970. They had a larger range and colonies supported more individuals than they do today. Thus they represent an appropriate population with which to establish a baseline GC level: one which characterizes low disturbance and an abundance of marmots. Any modern colonies that have significantly elevated GC levels compared to this baseline are those which are most likely to be disturbed by human activity which is a relatively novel threat on Vancouver Island. However, analysis of GCs through time cannot determine which disturbance affects marmots if many disturbances increased in frequency or scale during the same time period. Public roads and resource extraction both present a variety of challenges to wildlife, but both became prominent features of the landscape during the decades between 1910 and 1970. The relative importance of each of these potential threats must therefore be investigated using the extant population, which lives in colonies that are at various proximities to sources of human disturbance. Colonies that are in close proximity to a human activity which marmots perceive as a threat should have higher levels of cortisol than those colonies that are more distant. I used information about where marmot colonies are in relation to anthropogenic features of the landscape to determine if

human disturbance is related to hair cortisol levels between 2008 and 2016, with the goal of understanding the reason for marmots to have high GC levels in some colonies, but not others. [4]I measured the distance to roads and active cutblocks, which I expected to be negatively related to hair cortisol concentrations, and the distance to protected areas, which I expected to be positively related to hair cortisol concentration.

Marmots live in a dynamic environment and may encounter many challenges, some of which occur naturally. Therefore, an elevation in cortisol above historic baseline is not necessarily a result of human activity. To examine two alternative hypotheses for elevated cortisol levels, I also considered the effect of marmot demographics in each colony and the effect of weather conditions on marmots in each year of the study. In some social species, cortisol levels are high in large groups, possibly due to the increased likelihood of competition and agonistic encounters (Chapman *et al.*, 2007; Foley *et al.*, 2001). Marmots engage in agonistic behaviour such as chasing and fighting (Heard 1977) and evidence suggests that aggression has become more common as sociality has decreased in the species (Brashares et al. 2010). The optimal group size for Vancouver Island marmots is unknown, but behavioural studies suggest that individuals are territorial and groups are arranged in a dominance hierarchy, with adult males in the most dominant position (Heard, 1977). Therefore, it is possible that larger groups of marmots experience high levels of social stress, and that this relationship is modified by an individual's sex. I predicted that group size would be positively related to cortisol levels, regardless of modifications to the surrounding landscape and that cortisol levels would be higher in males than in females.

Certain abiotic conditions that arise due to weather events may also be closely related to cortisol levels. Bechshoft et al (2013) found that weather conditions attributable to the El Nino Southern Oscillation relate to hair cortisol levels in polar bears (*Ursus maritimus*), perhaps by

impacting food availability in an Arctic ecosystem. Marmots are most likely to be affected by high daytime temperatures as marmots have limited thermoregulatory capabilities (Armitage, 2013). In Alpine marmots, temperatures in excess of 25 degrees Celsius cause a decrease in activity (Turk and Arnold, 1988) and several marmot species have undergone range shifts in response to warming (Armitage, 2013). Average temperatures have increased by approximately 0.7 degrees Celsius in British Columbia since climate monitoring began (Hamann and Wang, 2006). It is likely that temperatures now exceed 25 degrees Celsius during more days per year than they did prior to the current period of climate warming. This could serve as a challenge for Vancouver Island marmots if they have limited ability to thermoregulate at temperatures in excess of 25 degrees. If warmer-than-average summer temperatures are related to elevated cortisol levels in Vancouver Island marmots, this would be easily detected using hair cortisol analysis, given that moulting occurs during the warmest time of year (Naughton, 2014). Therefore, I predicted that cortisol levels would be higher during years that had higher temperatures.

I tested cortisol levels in contemporary animals in contrast to a historic baseline to determine if cortisol has increased as the species has declined. Then I tested three possible explanations for a change in cortisol from the historic baseline. First, cortisol levels in Vancouver Island marmots are related to the type and proximity of human disturbance to the marmot's habitat. I selected only novel sources of disturbance that have become potential challenges for marmots in the past 50 years. Because some marmot colonies are purposefully protected from disturbance by laws and regulations on land use, I tested the possibility that protected areas are also related to cortisol levels. Second, cortisol levels are not related to human activity and are instead related to the size of the group living at the colony. This could explain a change in cortisol from the historic baseline if social behaviour in the species has changed over time and aggression has increased (Brashares *et al.*, 2010). Third, cortisol levels are related to high air temperatures. This would offer tenuous support to the

hypothesis that cortisol levels are elevated above baseline and would suggest instead that they vary considerably from year to year depending on abiotic conditions.

Methods

Sample collection

Historic samples were obtained from three different natural history collections: the Museum of Nature, Ottawa, Ontario (CNMNA 103333, 14088, 14089); the Beaty Biodiversity Museum, Vancouver, British Columbia (UBCBBM CTC M000928, M005861, M005862, M005863, M005864, M005866); and the Museum of Vertebrate Zoology, Berkeley, California (MVZ Mammals 12090, 12091, 12092, 12093, 12098, 12099, 12100). These specimens were found by querying the VertNet database (UBCCCM, MVZ Mammals) and the Museum of Nature database. Pelts were by plucking or clipping individual hairs within an 8 cm by 8 cm area. A minimum of 50 hairs were collected. The 8 cm by 8 cm area was selected to prevent sample collection from leaving a sparse or bald patch on the study pelts and diminishing their use in future.

Contemporary marmot samples were obtained from the Marmot Recovery Team. Marmots are routinely live-trapped and handled for translocation, veterinary care and other interventions (see (Bryant, 1996) for details). Samples were collected opportunistically from immobilized marmots by plucking or snipping close to the skin. Samples were collected between 2008 and 2016 and included individuals that were born and reared in the wild as well as individuals that had been born in captivity and were considered established in the wild after surviving more than one winter (see chapter two of this thesis for more details).

All samples were taken from the dorsal side of the body. An analysis of hair cortisol concentration by body region showed that the rump and back (both dorsal body regions tested) did not have significantly different hair cortisol concentrations in a captive population (see chapter one

of this thesis). Historic samples were taken consistently from the left dorsal area, near the left rump, using the tails and hind limbs of each pelt as landmarks. Opportunistically collected samples of live-trapped marmots were taken from the dorsum, not otherwise specified. All samples were collected in plain white envelopes or yellow coin envelopes and stored at room temperature until use.

Hair cortisol analysis

Cortisol was extracted in methanol and quantified using enzymeimmunoassay as described in the first chapter of this thesis. Inter-assay CVs were calculated by running external controls at 25% and 65% binding in duplicate on each plate. The CV for high control (25% binding) was 7.7% and for low control (65% binding) was 6.5%. Along with monitoring the CV of each duplicate, intra-assay CVs were further evaluated by loading a pooled fecal extract diluted to 50% binding repeatedly across the plate. For this assay, the intra-assay CV was 3.6%.

Demographic and geographic data

Demographic information about the marmot colonies was derived from the Marmot Recovery Team's handling summary. This dataset contains 2625 observations of 861 marmots that were handled between 2005 and 2016, including 312 free-ranging animals from 19 unique colonies. The number of unique marmots handled at each colony in a given year was used as an estimate of population size.

Geographic data for locations of active cutblocks and roads were derived from the Forest Tenure Cutblock Polygons dataset (FTA 4.0) from the British Columbia Ministry of Forest, Lands and Natural Resource Operations[5] (https://catalogue.data.gov.bc.ca/dataset/forest-tenure-cutblock-polygons-fta-4-0). The iMap BC online mapping application (https://maps.gov.bc.ca/ess/hm/imap4m/; powered by Esri ArcGIS) was used to measure the distance between each marmot colony and: the nearest paved road[6] (public, surfaced with asphalt);

the nearest unpaved road; and the closest edge of the nearest active cut block. The coordinates used by Heard (1977) were used to estimate the location of four marmot colonies. The other colony locations were estimated using the peak of the mountain where the colony was found. Geographic data about protected areas were available from the World Database on Protected Areas (http://www. protectedplanet.net/c/world-database-on-protected-areas) and the Global Forest Watch online mapping application (http://www.globalforestwatch.org/map) was used to measure the distance of each marmot colony from the closest edge of the nearest protected areas.

Meteorological data

Hourly air temperature data is recorded at high altitude weather stations by the government of British Columbia's River Forecasting Centre. The Wolf Creek weather station is located at 1422 m above sea level, approximately 35 km northwest of Mount Washington. Temperature data were available from 22 different years when maxima were recorded for at least 16 days in the month of July: 1988, 1990 and 1996-2015. The median maximum temperature measured each day during the month of July was used to determine if there was a difference in risk of heat stress during each year the marmots were active in the study. Marmots sampled in 2016 would have experienced the summer of 2015 and cortisol levels in their fur would correlate to temperatures that year, if a relationship were found to exist. [7]

Statistical analyses

All cortisol concentrations were transformed with the natural logarithm to achieve normality. See appendix A of this thesis for further information.

To test the possibility that marmot cortisol levels changed through time, data from free-ranging marmots was combined with data from several museums to calculate Pearson's product-moment correlation coefficient between sample year and hair cortisol concentration. [8]A total of 70

animals were included in the sample. Historic marmot samples from Douglas Peak, Green Mountain and Mt. Buttle were included in this analysis and represent marmot colonies from 1910 and 1929-1931.

Marmot samples were grouped[9] into distinct colonies to identify hair cortisol levels at particular geographic locations in time. Seven groups were selected *a priori* as they had sample sizes of 3 or more, contained at least one male and one female and all samples had been collected from one colony in a span of 3 years: Historic (Douglas Peak (1910), Green Mountain (1931)), early[10] Mt. Washington (2010-12), late Mt. Washington (2016), Haley Lake (2015-16), Mount Hooper (2016) and Knight Lake (2016). However, this left many observations from the southern part of the range absent from analysis. The Mount Washington observations and the historic marmots formed clearly delineated groups; as such, they were temporarily removed. The remaining observations represented small groups and individual marmots sampled across the range at different times. To investigate the structure of the data and identify any clusters, k-means cluster analysis [11](R package: cluster 2.0.6 (Maechler *et al.*, 2017)) was used to group observations around four centroids. This clustering accounted for 79% of the variation and revealed the following clusters: "Nanaimo Lakes" (all observations made in the southern part of the range prior to 2012); the Haley Lake group in addition to observations from Mount Moriarty and Steamboat Mountain ("HMS"); the Mount Hooper group in addition to observations from Castlecrag ("HC"); and the Knight Lake group in addition to observations from Mount McQuillan, Hooper North and Limestone Mountain ("KML"). See table 3.1 for an overview of clusters used in analysis. Cortisol levels in these groups were analyzed using one-way ANOVA and groups with significantly different HCC from the historic group were determined using Dunnett's test.

General linear mixed models were fitted to the data using the lmer function (R package: lme4 1.1-13 (Bates *et al.*, 2015)) with temperature, population size and geographic variables as predictors and individual cortisol level as the response. In the second chapter of this thesis, I found that male marmots had higher levels of cortisol than female marmots in captivity which suggests that male marmots may be more sensitive to environmental conditions than females. As such, sex was also included as a predictor in several models.

To test the primary hypothesis that cortisol is related to disturbance, nine models were created to examine the relationship between cortisol, proximity of a colony to a source of human disturbance and proximity to a protected area. Spatial data can be highly correlated. Exploratory analysis of the data revealed a strong Pearson product-moment correlation between the distance to the nearest protected area and the nearest paved road (0.54) and between the distance to the nearest protected area and the nearest active cutblock (-0.77). As such, distance to the nearest protected area was removed as a continuous predictor and was included as a categorical predictor (colony in a protected area: yes or no). The following models were created to test the first hypothesis:

M1: cortisol = cutblock + colony ID + □

M2: cortisol = unpaved road + colony ID + □

M3: cortisol = paved road + colony ID + □

M4: cortisol = cutblock * protected area + colony ID + □

M5: cortisol = unpaved road * protected area + colony ID + □

M6: cortisol = paved road * protected area + colony ID + □

M7: cortisol = cutblock * sex + colony ID + □

M8: cortisol = unpaved road * sex + colony ID + □

M9: cortisol = paved road * sex + colony ID + □

To test the alternate hypotheses that cortisol is better explained by demographic predictors, I created two additional models:

M10: cortisol = population + colony ID + □

M11: cortisol = population * sex + colony ID + □

Finally, to test the alternate hypothesis that cortisol is best explained by temperature, I created two additional models:

M12: cortisol = temperature + colony ID + □

M13: cortisol = temperature * sex + colony ID + □

A null model was created using the random effect of colony identity. All models were ranked using the second-order Akaike's Information Criterion (AICc) for small sample sizes. The model with the lowest AICc was selected as the best estimate of the true model. Following Brearley et al. (2012), AICc weights were calculated for each model and the AICc weights for models with like predictors were summed to give the relative importance of each predictor.

Results

There was a weak positive relationship (adjusted $R^2 = 0.269$; $F = 2.058$; $df = 24, 45$; $p = 0.0182$) between hair cortisol level and sample year and colony. There was no interaction between sample year and colony ($F = 0.3114$; $df = 4, 45$; $p = 0.869$). The mean hair cortisol concentration of the historic marmots was 1.14 ng/g hair (95% confidence interval: 1.038, 1.248). ANOVA revealed that colony had a significant effect on hair cortisol concentration ($F = 7.857$; $df = 6, 63$; $p < 0.001$; see figure 3.1). Among marmots sampled before 2012, the HCC in the Nanaimo Lakes group was 1.137 (95% CI: 1.027, 1.248) and in early Mt. Washington it was 1.359 (95% CI: 1.234, 1.484). Among marmots samples in 2015-16, the HCC in the Haley Lake group was 1.528 (95% CI: 1.442, 1.615), in the Knight Lake group it was (95% CI: 1.161, 1.454), in the late Mount Washington group it was

2.250 (95% CI: 2.049, 2.452) and at Mount Hooper-Castlecrag it was 2.338 (95% CI: 2.187, 2.490). Dunnett's test showed that late Mount Washington ($t = 5.021$, $p < 0.001$) and Mount Hooper-Castlecrag ($t = 4.554$, $p < 0.001$) had significantly higher HCC than historic levels. There was no obvious geographical clustering of high cortisol colonies (see figure 3.2) as two were found in the northern end of the range, and one was located in the southern end of the range, in close proximity to most of the low-cortisol colonies.

Two models had lower AICc values than the null model. The model with the lowest AICc was M2 which included distance to the nearest unpaved road as the only predictor. The coefficient estimate for the effect of unpaved road was -0.0931 ± 0.03266 (SE). The AICc weight of this model was 0.54. This model provided a better fit than M8, the model that included nearest paved road and sex as predictors (□ AICc: 2.54, AICc weight = 0.15). See Table 3.2 for a summary of all models evaluated. The sum AICc weights for the models that included unpaved road as a predictor is 0.74. This is 3.7 times higher than the weight of the next most important predictor, sex (0.2). The weights of the other predictors were 0.07 (protected area), 0.06 (active cutblock), 0.05 (paved road), 0.04 (temperature, population size). See Table 3.3 for a summary of the predictors. For a more detailed description of the predictor variables, see Appendix D.

Discussion

Most[12] contemporary Vancouver Island marmot colonies along with two historic colonies that existed in a time of greater marmot abundance and less human activity, cannot be distinguished by their hair cortisol concentrations. This suggests that there has not been a general increase in GCs as human disturbances have proliferated. This may be because the changes that have occurred on Vancouver Island in the past century have not been on a spatial scale that is large enough to impact the species at all colony locations. Many of the sources of disturbance measured were at a

considerable distance from marmot habitat. The average distance from a colony to a paved road was 10.6 km and from a colony to an active cutblock was 7.3 km. Additionally, the nearest paved road to several of the colonies was not a public highway or other road that might experience high-volume traffic, but a secondary road connecting small towns or villages; large urban or suburban population centres were so distant from marmot colonies that they were not even included in this study. It is likely that the remote nature of the meadows that marmots occupy protects them from most types of disturbance. It is also possible that modern marmots do not generally have elevated GC levels because many of the changes that have occurred on Vancouver Island over the past 100 years are not perceived as challenges. Marmots do not avoid roads when dispersing to new habitat (Bryant 1990) and several marmots have been observed near the Strathcona Parkway on Mount Washington (Jackson *et al.*, 2015), a paved public road with a moderate posted speed limit. Nor do marmots avoid cutblocks, albeit once industrial activity has ended; marmot will disperse to clear-cut areas and establish colonies there (Bryant, 1996). Either due to the spatially heterogeneous nature of human development that has spared a considerable amount of marmot habitat, or a high tolerance of anthropogenic modification to the landscape, many extant marmot colonies are living under conditions of low disturbance; there are far fewer colonies where disturbance may be high.

Marmot colonies at Mount Washington, Mount Hooper and Castecrag are all living in habitats that might be disturbed by human activity. The GC levels in these animals were significantly higher than those in the historic population and the best model of these data suggest that the cortisol level in an individual's hair can be predicted by the proximity of their colony to a road that has not been surfaced with asphalt ("unpaved"), such as a logging road. The negative relationship between unpaved roads and cortisol suggests that the closer a colony is to an unpaved road, the higher the cortisol levels are at that location. Taken together, these data suggest that the increase in GCs might have an anthropogenic cause. It could be that logging roads are a threat to Vancouver

Island marmots. The Castlecrag colony is within 1 km of an unpaved road and is the only colony in the study found within 1 km of an anthropogenic disturbance. The direct effects of roads and industrial activities have been shown to extend 250 to 800 m beyond the source of the disturbance (Forman, 2000; Potocnik and Poje, 2010), meaning that marmots may be directly disturbed by roads at this location. Although these roads are not public thoroughfares and may not see heavy traffic, they are likely accessed by industrial equipment as there are active timber harvest sites in the area, and heavy equipment traveling along these roads could cause noise pollution. Animals which rely on auditory communication are thought to be highly impacted by noise pollution (Ortega, 2012; Tennessen *et al.*, 2014). Marmots use alarm calls to alert conspecifics of predators and they use other vocalizations to communicate during social interactions such as playing or fighting (Blumstein, 1999). A multi-year study of marmot behaviour in the early 2000s found that social behaviour had changed considerably since it was first studied in the 1970s (Brashares *et al.*, 2010). Noise pollution may be able to considerably disrupt marmot social behaviour or alarm calling. An analysis of the type and amount of traffic utilizing these roads is beyond the scope of this study, but these results suggest that despite the seemingly remote nature of marmot habitats, some colonies may indeed be directly disturbed by human activity.

However, given that marmots do not show behavioural avoidance of roads (Bryant, 1998) and the other high-cortisol colonies, Mount Hooper and Mount Washington, are 3.6 km and 1.8 km respectively from the nearest unpaved road, it seems unlikely that road noise is a threat to marmots. However, in addition to being sources of noise pollution, roads can also serve as transportation corridors for predators. Both wolves (*Canis lupis*) and puma (*Felis concolor*) live on Vancouver Island and rely on black-tailed deer (*Odocoileus hemionus hemionus*) as primary prey species (Bryant and Page, 2005). But, as generalist predators, both will also exploit smaller prey (Spalding and Lesowski 1971; Wilson et al. 2004; Price et al. 2005). Marmots are found only at high altitudes (> 1000 m above sea

level, Bryant & Janz 1996) so terrestrial predators that hunt marmots are likely incurring some energetic costs to do so. One strategy that carnivores may use to offset this energetic expenditure is to walk along roads and trails to access marmot colonies (Bryant, 1998). Wolves and pumas are both known to use linear features of the landscape such as roads when seeking out prey (Dickie *et al.*, 2016; Dickson *et al.*, 2005) and they may be particularly useful when hunting at high altitudes (Whittington *et al.*, 2011). But they are less likely to use paved roads than trails, or other linear features with low human use (Dickson *et al.*, 2005; Northrup *et al.*, 2012; Whittington *et al.*, 2005). This may explain why unpaved roads and not paved roads are related to cortisol levels in marmots.

Predation is often cited as a significant cause of mortality among free-ranging marmots (Aaltonen *et al.*, 2009; Bryant and Page, 2005; Jackson *et al.*, 2016) which has given rise to the hypothesis that changing predator-prey dynamics on Vancouver Island are to blame for the marmot's decline (Janz *et al.*, 2000; Vancouver Island Marmot Recovery Team, 2008). Black-tailed deer populations on Vancouver Island decreased sharply in the late 1970s (McNay and Voller, 1995) and this may have caused wolves and pumas to exert novel pressure on Vancouver Island marmots during the 1980s and 1990s (Bryant and Page, 2005). With low reproductive rates (Bryant 2005), the species could have been driven to near-extinction by a sudden increase in predation (Bryant and Page, 2005). Black-tailed deer populations are now stable or increasing across Vancouver Island yet marmots are still at high risk of predation by terrestrial carnivores (Jackson *et al.*, 2016). It is possible that the creation of logging roads has led to a shift in predator behaviour that has endured despite the rebounding of other prey populations. Given the threat that predation poses to Vancouver Island marmots, it may be advisable to focus marmot recovery efforts on locations that are distant from linear features such as roads, regardless of how often they are accessed for human use.

I predicted that sex would interact with other factors to be an important predictor of hair cortisol, but I did not find strong evidence for this. The predictive value of the model which included distance to the nearest unpaved road declined when an interaction with sex was included indicating that the simplest model was the better model. It is possible that I failed to detect a true interaction between disturbance and sex because the effect size may have been very small and I had only three high-cortisol colonies in my sample. Small sample sizes are one of the many challenges to overcome when studying species-at-risk. Vancouver Island marmot populations have been small since the 1990s when they were first listed as an endangered species and this makes it difficult to sample sufficient animals to draw meaningful conclusions about their general biology. In order to overcome the problem of a small extant population I relied on archived samples that had been collected for genotyping purposes. This is an important advantage of using hair as a substrate to measure physiology, and the technique merits further research and attention. While some research teams do not have long-term archives of hair samples, museum collections often do; such samples can allow researchers to test differences in physiology that exist across spatial or temporal gradients. Not only does this represent a considerably large sample size, it can also allow researchers to make comparisons between the present day and the physiology of animals in earlier time periods. This may be of particular interest in understanding declines in species that have begun very recently.

In addition to a small extant population, most of the colonies are very remote and the Recovery Team has limited capabilities to sample marmots at each colony every year. Many of the samples used in this study came from marmots living on Mount Washington, the most accessible colony, which may have skewed the results to be representative of that location rather than representative of marmots in colonies across the range. It is possible that the results obtained from Mount Washington are confounded by other factors that influence cortisol levels. Mount Washington is where the Recovery Team's veterinary facility is located and is generally the initial

release site for marmots receiving a soft release from captivity (Jackson *et al.*, 2015; McAdie, 2004). Due to its proximity to the Mount Washington Alpine Resort, this site is the most accessible to researchers and marmots can be food provisioned here (Jackson *et al.*, 2015). Therefore, there is a high level of disturbance at this site, although it is unknown if the presence of researchers is perceived as a threat by these animals. It is also likely that the number of marmots living at Mount Washington is artificially large: there were 25 individuals living there in 2016 while most other colonies had less than 10. Arguably, it does not represent a natural population. However, the early Mount Washington marmots, who were subject to similar interventions and interactions with humans between 2010 and 2012, have significantly lower cortisol levels than the 2016 group. The elevated cortisol levels detected in the later Mount Washington group cannot be exclusively due to the singular nature of the site. These results should not be discounted but should perhaps be interpreted with some caution.

While elevated glucocorticoid levels may be indicative of poor living conditions, they are likely an adaptive response to such conditions (Boonstra, 2013). It is not clear if chronically elevated glucocorticoid levels in free-ranging or natural populations is associated with poor survival or reproduction; many studies have found a positive relationship between glucocorticoid levels and fitness in wild animals (Bonier *et al.*, 2009). The levels at which glucocorticoids become detrimental, or the period over which this elevation must occur, is not generally known for most species and two populations with different glucocorticoid levels could still have similar fitness (Dantzer *et al.*, 2014). Furthermore, studies have found elevated glucocorticoid levels among populations living in rural environments compared to those living in urban environments – evidence that high GC levels do not always indicate that a population is under threat from human activity (e.g., French et al. 2008). Although the cortisol levels measured in marmots at Mt. Hooper, Mt. Washington and Castlecrag are significantly higher than historic levels, I do not know if the marmots in these colonies have

lower fitness than their conspecifics at low-cortisol colonies. Further research is necessary to understand the consequences of elevated cortisol levels in this species. Nonetheless, if these habitats present marmots with more challenges then other habitats on Vancouver Island, it may be advisable to focus repopulation efforts on colonies where marmots will face fewer threats. Marmots may be able to survive in these colonies due to the adaptive benefits of a strong adrenal response, but when decisions are made about where reintroductions and translocations are to take place, it may be advisable to release marmots in habitats where such a response is unnecessary.

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Table 3.1 The marmot clusters used to identify colonies with elevated hair cortisol concentration using one-way analysis of variance (ANOVA). Historic, early Mount Washington, late Mount Washington, Haley Lake, Knight Lake and Mt Hooper were selected *a priori* to provide colony-level estimates of cortisol concentrations; k-means cluster analysis was used to investigate grouping of all additional observations (*) and produced clustering around 4 centroids: Nanaimo Lakes, Haley Lake, Knight Lake and Mt. Hooper.

Cluster	**Colonies**	**Years**	**Males**	**Females**	**Total**
Historic	*Douglas Peak Green Mtn.* *Mt. Buttle*	1910, 1929, 1931	8	7	15
Nanaimo Lakes*	Haley Lake Mt. Buttle P Mtn. Butler Peak Green Mtn. Mt. Moriarty Steamboat Mtn.	2008, 2011, 2012	4	6	10
Early Mt. Washington	*Mt. Washington*	2010, 2012	2	4	6
Late Mt. Washington	*Mt. Washington*	2016	7	10	17
Haley Lake	*Haley Lake* Mt. Moriarty* Steamboat Mtn.*	2015, 2016	2	4	6
Knight Lake	*Knight Lake* Mt. McQuillan* Hooper North* Limestone Mtn.*	2016	4	3	7
Mt. Hooper	*Mt. Hooper* Castlecrag*	2016	6	3	9

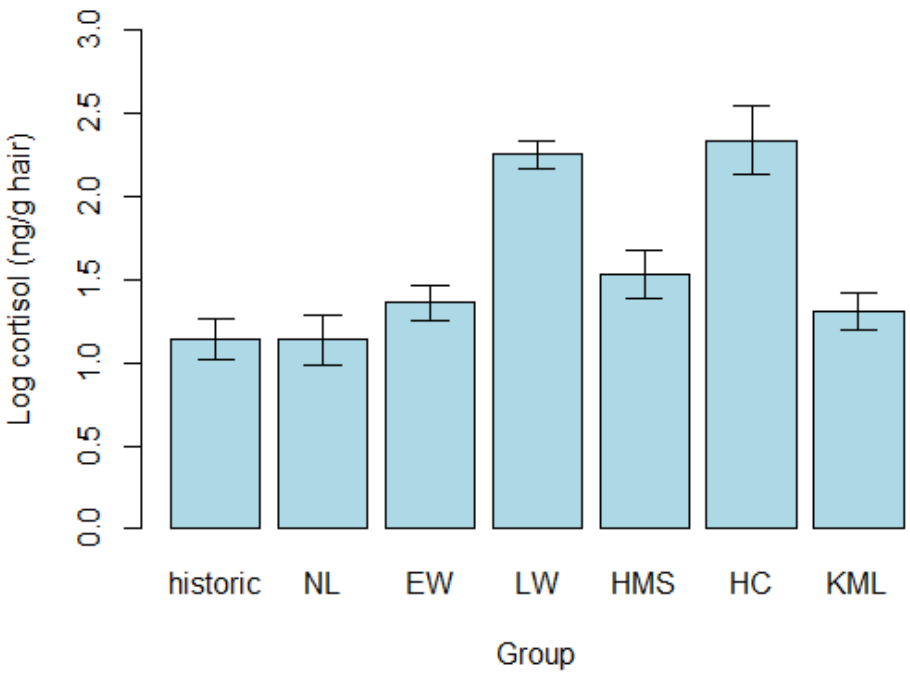

Figure 3.1 The mean hair cortisol concentration (HCC) in seven marmot groups: historic marmots (southern colonies, 1910-1931), Nanaimo Lakes (NL – southern colonies, 2008-12), early Mt. Washington (EW – northern colony, 2010-12), late Mt. Washington (2016), Haley Lake (HMS – plus two additional southern colonies, 2016), Mt. Hooper-Castlecrag (HC – 2016) and Knight Lake (KLM – plus three additional southern colonies, 2016). Late Mt. Washington and Mt. Hooper-Castlecrag had significantly elevated hair cortisol levels compared to the historic baseline ($F = 7.857$; $df = 6, 63$; $p < 0.001$). HCC is given as the log cortisol (ng) per gram of dry hair. Error bars indicate 95% confidence interval.

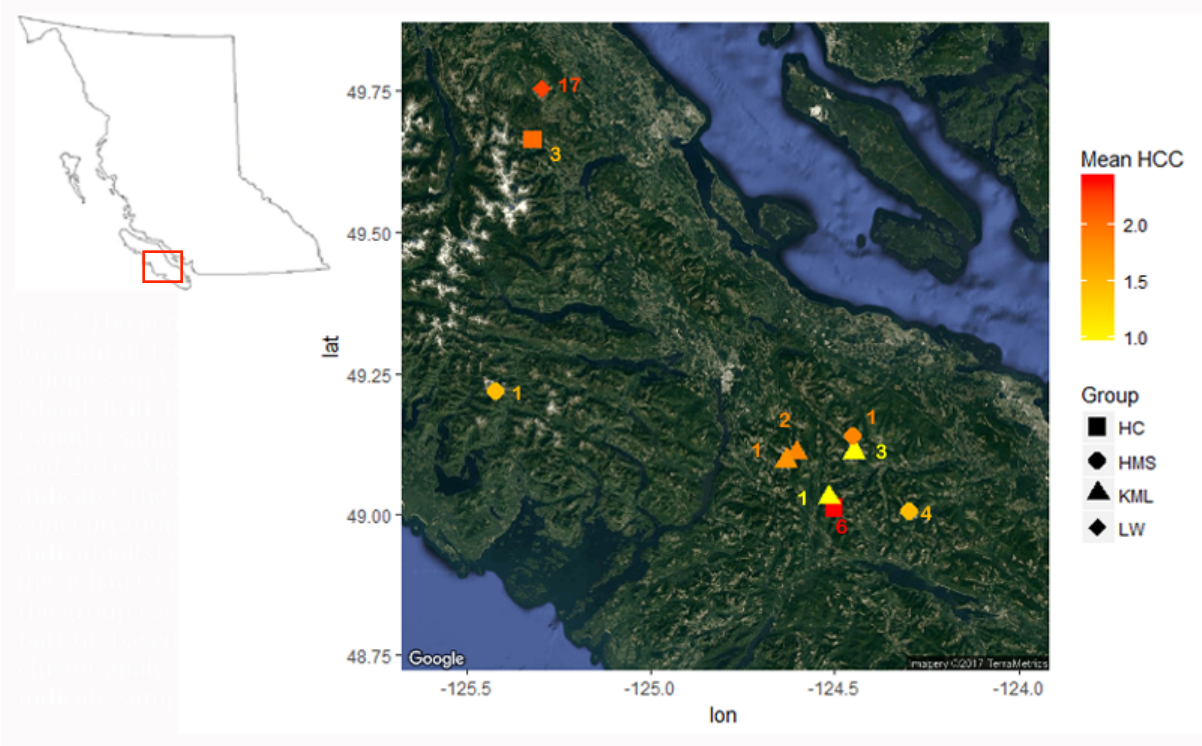

Figure 3.2 Map showing generalized locations of all marmot colonies sampled during the 2016 field season on Vancouver Island in British Columbia, Canada. Colour indicates the hair cortisol concentration (HCC) in marmots at that location. Shape indicates the cluster used to calculate ANOVA. Number indicates the sample size at that location. HCC is given as log cortisol (ng) per gram of dry hair. HC – Mount Hooper-Castlecrag; HMS – Haley Lake, Mount Moriarty, Steamboat Mountain; KML – Knight Lake, Mount McQuillan, Hooper North, Limestone Mountain; LW – Mount Washington.

Table 3.2 The generalized linear mixed models used to model hair cortisol concentration in free-ranging Vancouver Island marmots (n=54). Models are ranked by second order Aikaike's Information Criterion (AICc). The null model includes colony identity as a random effect.

Model	Predictors	AICc	□ AICc	AICc weight
M2	distance to an unpaved road	125.45	0	0.54
M8	distance to an unpaved road * sex	128.00	2.54	0.15
null		129.19	3.74	0.08
M5	distance to an unpaved road * protected area	130.3	4.85	0.05
M10	population size	131.14	5.69	0.03
M3	distance to a paved road	131.26	5.81	0.03
M1	distance to an active cutblock	131.49	6.04	0.03
M12	max. temperature in July	131.49	6.04	0.03
M7	distance to an active cutblock * sex	132.02	6.57	0.02
M13	max. temperature in July * sex	133.36	7.91	0.01
M9	distance to a paved road * sex	133.77	8.32	0.01
M11	population size * sex	133.77	8.32	0.01
M6	distance to a paved road * protected area	134.28	8.83	0.01
M4	distance to an active cutblock * protected area	134.66	9.21	0.01

Table 3.3 The predictors used to model hair cortisol concentration in free-ranging Vancouver Island marmots. The sample includes 54 marmots from 14 distinct colonies. Predictors are ranked by the sum of the AICc weights of each candidate model which included that predictor

Continuous Predictors	**Min**	**Max**	**Mean**	**SD**	**Rank**	**AICc weight**
distance to an unpaved road (km)	0.54	12.1	4.14	3.84	1	0.74
distance to an active cutblock (km)	3.10	14.2	7.83	3.64	4	0.06
distance to a paved road (km)	1.90	17.1	10.6	5.40	5	0.05
population size	1	25	10.1	7.82	6	0.04
max. temperature reached in July (ºC)	20.4	32.3	24.5	2.33	6	0.04
Categorical predictors						
sex	**males**: 24		**females**: 30		2	0.20
protected area	**yes**: 9		**no**: 45		3	0.07

Appendix D: The predictors of hair cortisol levels in individual marmots 2008-2016.

The mean maximum temperature recorded in July at Wolf River between 1988 and 2015 was 24.5 degrees Celsius. The warmest year on record was 2007 when the maximum temperature was 32.3 degrees Celsius and the temperature routinely exceeded 25 degrees Celsius. The coolest year on record was 2011 when the temperature only exceeded 20 degrees Celsius on one day (July 25, 2011). During ten of the years sampled, the temperature never exceeded 25 degrees, including the majority of the years included in this study: 2010, 2011, 2012, 2013 and 2015. See figure D.1.[13]

There were 8 different unpaved roads that came in close proximity to marmot colonies[14]. The average distance from a colony to an unpaved road was 5.31 +/- 3.84 (SD) km. Castlecrag was closest to an unpaved road (0.54 km) and Butler was farthest from an unpaved road (12.1 km). There were 6 different paved roads that came in close proximity to marmot colonies, two of which were provincial highways (4 and 19) and one of which was the Strathcona Parkway which connects the Mount Washington resort area with the city of Courtenay. The average distance from a colony to a paved road was 10.6 ± 5.40 km. Mount Washington was closest to a paved road (1.90 km) and Castlecrag was the farthest from a paved road (17.1 km). There are 5 different protected areas in close proximity to marmot colonies, including 3 provincial parks. Haley Lake, Green Mountain and Steamboat Mountain are all located within protected areas while Limestone Mountain was located farthest from a protected area (19.1 km). There were 7 active cutblocks in close proximity to marmot colonies. The average distance from a colony to an active cutblock was 7.83 ± 3.64 km. Mt. McQuillan was located closest to an active cutblock (3.10 km) while Castlecrag was located farthest from an active cutblock (14.2 km).[15]

Marmot population estimates were small at most colonies. The median population size was 4 marmots per colony with a median female population of 2 and a sex ratio of 0.5. The Mount

Washington colony supported the largest population in 2012 (25 animals) and was large in other years sampled (8 animals in 2010, 18 animals in 2016). Mount Moriarty in 2012 and Hooper North in 2016 had very small populations (only 1 animal trapped at each location).

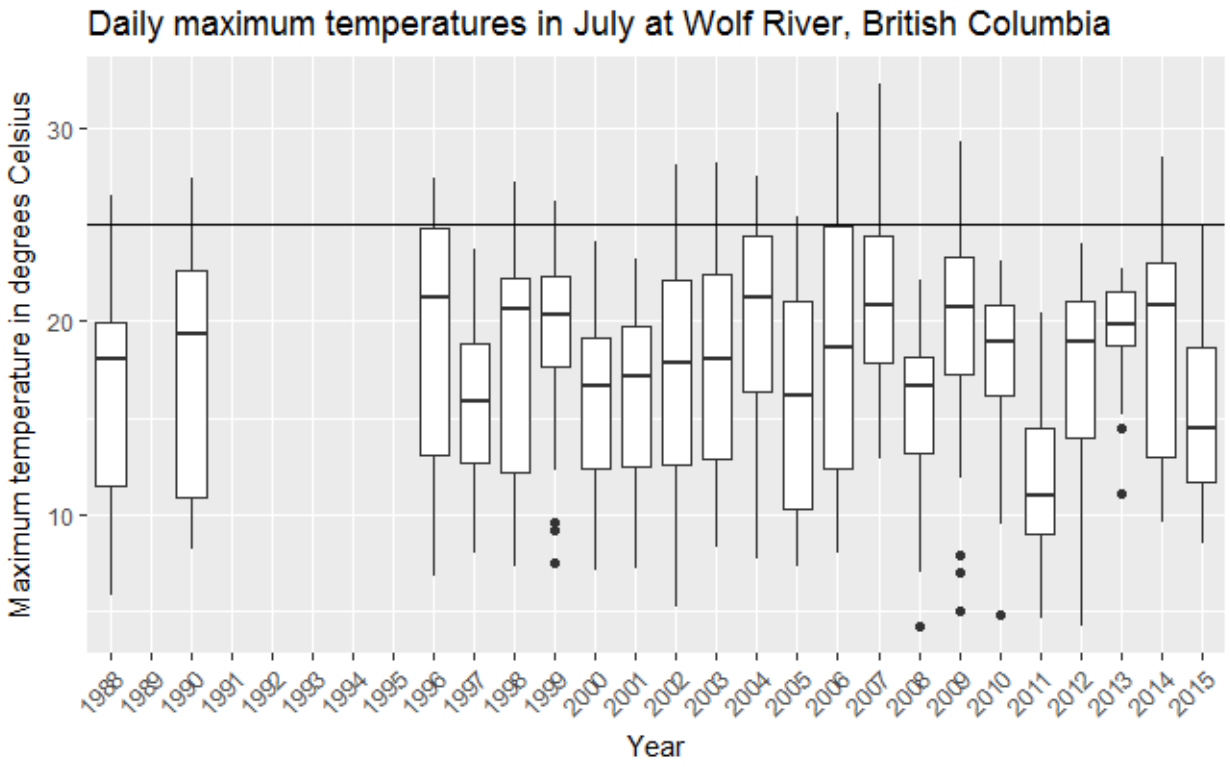

Figure D.1 The daily maximum temperatures recorded during the month of July at Wolf River on Vancouver Island, British Columbia

GENERAL DISCUSSION

The Vancouver Island marmot has been the subject of study for an entire generation of biologists and the subject of a sustained and intensive conservation effort for two decades. In this time, many hypotheses have been put forward to explain the species decline and its slow recovery which has faced numerous setbacks (Bryant et al. 2002; Bryant & Page 2005; Brashares et al. 2010). One of the problems facing researchers and conservation professionals alike is an uncertain understanding of the species ecology under natural conditions. It is difficult to determine what a stable marmot population might look like as both Vancouver Island and the marmots themselves may be fundamentally different today than they were in the past (Bryant 1998). The rapid increase in greenhouse gases in the atmosphere, the exponential growth of the human population and the development of land for its purposes have all contributed to rapid global change since the invention of the internal combustion engine (Steffen et al. 2011). It is reasonable to assume that these large-scale factors play a role in changing ecosystems at a pace which far exceeds many species ability to adapt (Dirzo et al. 2014). During this same time period, it is likely that marmots have also undergone dramatic change. The Vancouver Island marmot population reached a low of a few dozen adults in 2003 and this likely resulted in a loss of genetic diversity and a concomitant decline in fitness (Bryant & Page 2005; Kruckenhauser et al. 2009). Moreover, marmots have been bred in captivity since the late 1990s as a conservation measure (McAdie 2004). This practice has the potential to modify gene flow (Araki et al. 2007), allowing deleterious alleles to proliferate unhindered by sexual selection or by exposing organisms to the selective pressures of the captive environment rather than natural selection in the wild (Lynch & O'Hely 2001; Wedekind 2002). Yet, for critically endangered species, many of the ecological data points necessary to understand what good fitness resembled prior to grave population decline are lacking. New strategies are necessary to fill in these gaps.

Museum collections contain millions of specimens, presciently collected decades or centuries ago, which can provide physiological data from animals living in the earlier days of the industrial revolution. For instance, stable isotope analysis using hair or feathers can offer insight into changes in food intake (Hilderbrand et al. 1996; Blight et al. 2015) and heavy metals analysis can provide a retrospective calendar of intergenerational exposure within a population (Bocharova et al. 2013). My results show that it is also possible to quantify cortisol levels using hair samples that have been housed in museum collections. This makes it possible to contrast cortisol levels in populations living in different locations, during different time periods, and under different conditions, to investigate how cortisol levels correlate with anthropogenic activities. Thus, although I cannot quantify marmot fitness in the early 1900s directly using data regarding their reproduction and survival, I was able to establish a cortisol level that may provide an indirect measure of the magnitude of challenges animals faced under normal conditions, in natural habitat. Using this as a baseline, I found that only three contemporary marmot colonies deviated significantly from this level, with high cortisol recorded at Mount Washington, Mount Hooper and Castlecrag.

The clear distinction between individuals living in high-cortisol and low-cortisol colonies allowed me to test some hypotheses as to why cortisol levels may be elevated in some geographic locations and in some years, but not in others. I expected cortisol levels to be higher in marmot colonies that were in closer proximity to anthropogenic features of the landscape based on the assumption that human disturbance is perceived as a threat by this species and is therefore a potential barrier to species recovery. I used demographics and spatial variables, as well as a weather variable to determine if anthropogenic changes to the landscape are correlated to cortisol, or if cortisol levels were better explained by natural phenomena. I found that cortisol correlated with one anthropogenic factor, the proximity of unpaved roads to marmot colonies. This landscape feature may be a threat to marmots because predators use logging roads to access marmot colonies (Bryant

1998). Predation is often cited as the primary impediment to marmot recovery and survival (Bryant 1996; Bryant & Page 2005; Aaltonen et al. 2009), but it is difficult to find evidence for the changes that have driven naturally-occurring predators to exert unsustainable pressure on Vancouver Island marmots in recent decades. Brashares et al. (2010) found that anti-predator behaviour in the wild has changed since marmot behaviour was first described in the 1970s which suggests that marmots have become more susceptible to predation. Bryant (1999) documented changes driven by timber companies during the 1970s and 1980s on Vancouver Island, but was not able to demonstrate a connection between these changes and marmot fitness. With this study, I was able to show one way in which landscape change may be related to predator behaviour and marmot survival.

It is possible that marmots at these high cortisol colonies do not experience compromised fitness. I do not have direct experimental evidence to show that elevated cortisol levels in Vancouver Island marmots suppress reproduction or that they cause a decline in survivorship. But studies of closely related alpine marmots, *Marmota marmota* and yellow-bellied marmots, *Marmota flaviventris* show an inverse correlation between glucocorticoid levels and reproductive success, perhaps because GCs play an important role in mediating energy budgets and adequate energy stores are necessary to reproduce successfully (Hackländer et al. 2003; Blumstein et al. 2016). Vancouver Island marmots are a critically endangered species whose numbers have reached lows of approximately 30 individuals as recently as 2003 (Bryant & Page 2005). It is certain that the fitness of many marmots has decreased since the early 1900s when populations were larger and more widespread (Bryant & Janz 1996) It is therefore plausible that high cortisol colonies are at increased risk of reproductive failure and demographic collapse compared to the other colonies involved in this study, in which cortisol levels were at the historic baseline. It is advisable that further studies are conducted at these locations to better understand why cortisol levels are so elevated here and what the consequences are at the organismal and population level.

In addition to studying marmots under natural conditions, I also wanted to contribute to a broader understanding of the effects of captivity and captive breeding in this species. Many of the marmots living in the wild today were once housed in captivity. Captivity and release from captivity can both be challenges to wildlife (Teixeira et al. 2007; Morgan & Tromborg 2007), thereby possibly compounding the effects of disturbance at marmot colonies where many of the members are captive-reared. Moreover, a sustained stress response could undermine the goals of a captive breeding program which is considered to be critical to the species survival (Roach 2017). Although by many metrics the program has been successful, reintroduced marmots still have lower survival rates than those that are reared in the wild (Aaltonen et al. 2009; Jackson et al. 2016). Furthermore, captive breeding pairs have not been able to breed at rates that are any higher than wild pairs, although all adults are food provisioned and given uninterrupted access to a mate (Bryant 2005; Jackson et al. 2015). My results showed that marmots in captivity had lower cortisol levels than wild marmots. This means that it is likely no more challenging to live in captivity than in the wild and there is no evidence that captivity represents an additional threat to the persistence of the species. My results also showed that marmots had high cortisol levels following their initial release from captivity, but that marmots who survived their first winter in the wild persisted with significantly lower cortisol levels. These results offer a possible explanation for the low survival that Jackson et al (2016) observed among newly released marmots but which they did not observe in "established" marmots, or those which had had spent more than a year in the wild.

In my study of body regions, I found that hair cortisol concentration varied considerably between body regions; it was particularly high in the hind limb and quite variable in the chest. It is difficult to speculate about the causes of these differences because the mechanism through which cortisol is incorporated into hair is still unknown (Keckeis et al. 2012; Cattet et al. 2014). The structure and function of hair follicles should be the subject of further study so that the

physiological mechanisms that underpin this type of analysis can be more clearly defined (e.g., Ito et al. 2005). This is especially true among non-human mammals which display remarkable diversity in hair types and growth patterns (Ling 1970; Khan et al. 2014). A year-long observational study in a species which moults seasonally, as well as a long-term validation study using chronic adrenal challenge and sampling from multiple body regions, would both be useful in interpreting the results of chapter 1 and improving hair analysis as a technique for quantifying GCs in fur-bearing mammals.

When Heard first described the social behaviour of Vancouver Island marmots in 1977, very little else was known about them. Since that time, many studies have focussed on the demography of the species (Bryant & Janz 1996; Bryant 1996; Bryant & Page 2005; Brashares et al. 2010). This is undoubtedly because the survival of each individual is of considerable and immediate importance to rescue the species from the threat of extinction. However, as Bryant aptly stated in 1998, "organisms may not necessarily be most numerous in habitats where they are most successful in demographic terms… For many species it is therefore extremely dangerous to equate high abundance with habitat quality, or to equate low abundance with habitat unsuitability." While marmot counts are valuable data, they are not likely sufficient to provide explanations for the decline of the species in recent decades, nor to provide solutions. The answers probably lie in the microscopic processes of marmot biology. With a few important exceptions (Bryant & McAdie 2003; Kruckenhauser et al. 2009), marmot physiology remains largely unknown including immune function and endocrine physiology. It is therefore plausible that wildlife managers are lacking important criteria for assessing habitat quality and risk. One of my goals was to describe an approach that may link individual physiology with conditions in which marmots live to facilitate management decisions and describe conditions where marmots should be assessed for fitness consequences.

Although the nature of the relationship between cortisol levels and fitness and between cortisol levels and habitat quality have not yet been defined in Vancouver Island marmots, my research is an important first step toward an understanding of these relationships and has the potential to be valuable in the management of this species. Addressing this knowledge gap represents an important role for zoos in the present era. Zoos house easily-accessible populations of endangered species in controlled environments. While data from these populations should be interpreted cautiously, data derived from zoo populations can nonetheless provide insight into basic biology (Miller et al. 2004). This is of the utmost importance in critically endangered species whose populations may be too small to test hypotheses or too remote to be adequately sampled. The same can be said of natural history collections, which may also provide a unique type of data that provides insight into the past (Miller et al. 2004). The planet's vertebrate species have declined in abundance by 25% since the 1970s (Dirzo et al. 2014). Maintaining biodiversity and recovering populations may seem an insurmountable task. The importance of stakeholder engagement and collaborative approaches have been much touted in the conservation community (Waylen et al. 2010). The ever-increasing need for data to improve outcomes for species-at-risk will similarly require creative approaches and extensive collaboration between wildlife managers and museums, zoos and other research institutes (Fernandez & Timberlake 2008).

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